Conformational dynamics of P-glycoprotein in lipid nanodiscs and detergent micelles reveal complex motions on a wide time scale

Mavis Jiarong Li, Miklos Gutman, and William M. Atkins

From the Department of Medicinal Chemistry, University of Washington, Seattle, Washington 98195-7610

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P-glycoprotein (P-gp) is a highly substrate-promiscuous efflux transporter that plays a critical role in drug disposition. P-gp utilizes ATP hydrolysis by nucleotide-binding domains (NBDs) to drive transitions between inward-facing (IF) conformations that bind drugs and outward-facing (OF) conformations that release them to the extracellular solution. However, the details of the protein dynamics within either macroscopic IF or OF conformation remain uncharacterized, and the functional role of local dynamics has not been determined. In this work we measured the local dynamics of the IF state of P-gp in lipid nanodiscs and in detergent solution by hydrogen–deuterium (H/D) exchange MS. We observed “EX1 exchange kinetics,” or bimodal kinetics, for some peptides distributed in both NBDs, particularly for P-gp in the lipid nanodiscs. Remarkably, the EX1 kinetics occurred on several time scales, ranging from seconds to hours, suggesting highly complex, and correlated, motions. The results indicate at least three distinct conformational states in the ligand-free P-gp and suggest a rough conformational landscape. Addition of excess ATP and vanadate, to favor the OF conformations, caused a generalized, but modest, decrease in H/D exchange throughout the NBDs and slowed the EX1 kinetic transitions of several peptides. The functional implications of the results are consistent with the possibility that conformational selection provides a source of substrate promiscuity.

The membrane transporter P-glycoprotein (P-gp) plays a critical role in disposition and distribution for a wide range of therapeutic drug classes and it is a major determinant of cancer cell drug resistance (1–3). Among the complex properties of P-gp, the mechanisms by which it achieves its extraordinary substrate promiscuity have not been fully understood. P-glycoprotein is a member of the ABC transporter family (ATP Binding Cassette), which includes many structurally related transporters that utilize ATP hydrolysis to transport lipophilic substrates. P-gp includes 12 transmembrane helices (TMHs) that comprise the drug-binding site and two nucleotide-binding domains (NBD) that alternately bind and hydrolyze ATP (4–6). The linear sequence of P-gp includes an N-terminal transmembrane domain containing six TMHs collectively referred to as “transmembrane domain 1” (TMD1), followed by NBD1, a linker region, six additional TMHs, collectively called “transmembrane domain 2” (TMD2), and a second NBD (NBD2). The three-dimensional structures of murine and Caenorhabditis elegans orthologs have provided a valuable model for the human P-gp, for which no crystal structure is available (Fig. 1, mouse P-gp structures).

The available crystal structures from multiple homologous ABC transporters, including Sav1886 and MsbA (7–9), combined with cryo-EM structures, DEER studies with spin-labeled variants, and FRET with fluorescently-labeled NBDs, suggest that P-gp undergoes large-scale opening and closing motions, between an inward-facing (IF) conformational ensemble and outward-facing (OF) conformational ensemble (10–12). For some ABC transporters, nucleotides drive the formation of a stable OF conformation with the NBDs in intimate contact. In addition, the transition state for ATP hydrolysis can be mimicked by addition of ATP, or ADP, and vanadate, which “traps” a quasi-stable OF conformation (13, 14). In contrast to Sav1886 or MsbA, however, recent data suggest that the IF to OF conformational transition of P-gp is less tightly coupled to nucleotide binding (15) or hydrolysis; even in the presence of excess nucleotide and vanadate, P-gp retains a substantial population of IF states with varying degrees of separation between NBDs (16, 17). The emerging model suggests that drug-free P-gp samples a wide range of IF conformations that could bind different substrates or inhibitors, even with nucleotide bound. Apparently, the drug-free P-gp explores an expansive conformational landscape with a dynamic equilibrium of states defined by different inter-NBD distances.

However, little is known about this landscape or the local conformational status of discrete regions within either the IF or OF ensembles. Molecular dynamics simulations and X-ray structures suggest that, in addition to the large amplitude motions associated with switching between IF and OF states, some regions undergo local fluctuations in secondary structure,
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Table 1

| Percent coverage of major domains of P-gp in detergent and nanodiscs | P-gp in detergent-lipid micelles | P-gp nanodiscs |
|---|---|---|
| Overall coverage (%) | 40.1 | 37.7 |
| TMD1 coverage (%) | 15.6 | 13 |
| TMD2 coverage (%) | 19 | 15.7 |
| NBD1 coverage (%) | 76.2 | 73.8 |
| NBD2 coverage (%) | 64.2 | 62.7 |
| Redundancy | 2.2 | 1.55 |
| Total peptides | 91 | 59 |
| Bimodal peptides (observed) | 4 | 15 |

including “hinge regions” in the TMHs (12, 18, 19). It is possible that low amplitude, local, transitions in these hinge regions could be propagated to the NBDs to account for the wide range of inter-NBD distances. However, no experimental methods have revealed specific conformational changes within the IF ensemble that could participate in the recruitment of substrates or coupling of binding and ATP hydrolysis. In fact, no information is available concerning the time scales for conformational relaxation within the ensemble. In addition, the structural models are obtained in the absence of a membrane, so the effects of a lipid bilayer on the conformational status remain uncharacterized.

To map the local dynamics of P-gp with increased spatial and temporal resolution, we have performed hydrogen–deuterium (H/D) exchange MS (H/DX MS) with P-gp in either lipid nanodiscs or in a lipid/detergent solution. The results indicate complex local dynamics superimposed on the IF conformational ensemble. Interestingly, the dynamics include motions on a remarkably wide range of time scales.

Results

Comparison of P-gp in nanodiscs and in lipid/detergent micelles in ligand-free and vanadate-trapped states

H/DX MS was performed with P-gp reconstituted in either DMPC nanodiscs or in detergent/lipid micelles, using membrane scaffold protein 1D1 (MSP1D1) as described under “Experimental procedures.” For each case, at various times after dilution into buffer containing D₂O, the exchange reactions were quenched, the protein was digested, and peptides were analyzed for deuterium uptake. The recovered observable peptides spanned 37.7 and 40.1% of the total protein primary sequence for P-gp in nanodiscs versus P-gp in detergent, respectively. As expected this mainly included peptides in the NBDs, covering 68.2 and 70.3% of the NBDs for the P-gp in nanodiscs versus in detergent, respectively (Table 1 and Fig. 1). Thus, the H/DX provides a good characterization of the backbone dynamics for the NBDs, with additional information about a few peptides in the TMHs.

Whereas all experiments with P-gp nanodiscs were run in parallel, so that there are no differences in deuterium loss during analysis (back exchange), the experiments with P-gp in micelles were performed separately and can only be qualitatively compared with the experiments with nanodiscs. Therefore, we adopt a conservative approach and do not quantitatively compare results in detergent micelles to nanodiscs samples. Regardless of this, the results clearly demonstrate that the deuterium exchange behavior is qualitatively very similar in the two environments.

Exemplary deuterium uptake profiles for a few peptides from P-gp in micelles are shown in Fig. 2. Uptake profiles for all peptides are included in the supporting Fig. S1. The deuterium uptake profiles are consistent with the structure of P-gp. For example, peptide 1 (residues 69–78) is in the membrane-embedded TMH1 and exchanges deuterium very slowly, whereas peptide 2 (residues 631–647) is in the unstructured linker region between the two halves of P-gp and exchanges deuterium very quickly. Other observed peptides have a range of deuterium exchange properties and some are discussed below.

The uptake profiles also reveal differential sensitivity of the peptides to vanadate trapping, as elaborated below.

A summary of all deuterium uptake data for peptides throughout the P-gp sequence is provided in Fig. 3. The fractional incorporation of deuterium into all recovered peptides at varying times is shown in Fig. 3A for the nucleotide-free P-gp as well as for ATP/vanadate-trapped P-gp (Vi Trapped in Fig. 3A) in both nanodiscs and detergent micelles. The individual points represent the midpoint of each observed peptide and report the percent of amide protons that have been exchanged for that peptide. Note that the density of points within the sequences corresponding to the TMHs is much lower than the density of points in the regions corresponding to NBDs. This reflects the lower recovery of peptides in the TMHs. The “butterfly plots” in Fig. 3A indicate only modest changes in H/D exchange upon vanadate trapping (lower half of each butterfly plot) compared with the apo-P-gp (upper half of each butterfly plot). The differences are most easily identified in the difference plots in Fig. 3B, where positive values indicate peptides with lower deuterium exchange in the vanadate-trapped samples compared with apo samples and negative values indicate greater exchange in the vanadate-trapped protein. These plots demonstrate that the relative deuterium exchange rates of peptides in different locations are very similar for P-gp in detergent versus in nanodiscs with a few notable differences. Furthermore, sequences
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Throughout both NBDs become modestly less susceptible to exchange upon vanadate trapping, and the overall differences are slightly larger for the P-gp in detergent than in nanodiscs.

A few specific peptides are of particular interest. It is notable that intracellular loops (ICL) ICL1, ICL3, and ICL4 that are thought to mediate coupling between drug-binding sites in the TMHs (18, 20, 21) and the NBDs (peptides from ICL2 were not observed) are also protected from exchange upon vanadate trapping, indicating that their local conformation is altered, as expected for a greater population of the OF conformations upon Vi trapping (Fig. S2). These ICLs exhibit a greater sensitivity to vanadate trapping in detergent than in nanodiscs. Consistently in both detergent and lipid environments, ICL4 is the most exchange-protected in the presence of vanadate, followed by ICL1.

The peptide-(69–78) in TM1 (peptide 1 in Fig. 2) is notable and provides a useful benchmark for the behavior of the TMHs. The recovery of peptides in the TMHs is very low, as expected because they are hydrophobic and likely depleted during sample clean-up, but it is striking that among the peptides recovered for the P-gp sequence, this peptide is one of only two peptides that exhibits an increase in deuterium exchange upon vanadate trapping. The increase in deuterium exchange is very modest but it was consistently observed in three separate experiments (see supporting Fig. S2). This is consistent with an increase in the contribution of the OF state upon vanadate trapping with corresponding movements of TM1 or neighboring TM helices, as suggested by cross-linking experiments and calculated solvent-accessible surface area changes (17, 22).

Another region that consistently shows a modest increase in deuterium exchange upon vanadate trapping is near the D and H loops of NBD2, but not observed in NBD1. In fact, this effect in NBD2 emphasizes the asymmetric effects of vanadate trapping that are well-documented (15, 17, 23). Specifically, the Walker A (WA) peptide and the Q loop in NBD1 undergo a larger decrease in solvent accessibility with vanadate trapping compared with NBD2, and in general, NBD1 is more sensitive to ATP/vanadate than NBD2. These results for the P-gp in nanodiscs and in detergent, in the presence and absence of ATP/vanadate, are superimposed on the three-dimensional structure of murine P-gp in Fig. 3, C and D.

Direct observation of conformational relaxations, through EX1 kinetics

Close examination of the time course of deuterium exchange revealed a large number of peptides with time-dependent bimodal mass envelope distributions indicative of "EX1 kinetics." Unlike the more commonly observed EX2 kinetics, where amide protons exchange gradually in an uncorrelated manner, EX1 behavior arises from correlated protein motions that occur more slowly than amide exchange (24). The EX1 kinetics can provide a direct measure of conformational transitions, in contrast to the EX2 kinetics that report on deuterium exchange of a single conformation or rapidly interconverting conformations. A total of 4 peptides exhibited varying degrees of mixed EX1/EX2 kinetics for P-gp in detergent and an additional 11 peptides demonstrated this behavior in nanodiscs, suggesting that the lipid bilayer slows down local dynamics that are otherwise too fast to observe in the detergent preparations, or the membrane induces additional local structure that slowly "opens." The deuterium uptake data for exemplary mixed EX1/EX2 behavior are shown in Fig. 4. The widespread distribution of peptides with EX1 kinetic behavior throughout the NBDs, particularly for P-gp in a lipid bilayer, suggests a wide range of conformational diversity on biologically relevant time scales (Fig. 4B). It is particularly striking that the EX1 kinetics occur on a such wide range of time scales for different peptides; some
Figure 3. H/DX comparison of apo P-gp versus vanadate trapped P-gp in detergent/lipid micelles or nanodiscs. A, butterfly plots showing hydrogen–deuterium exchange profiles of P-gp in either micelles (top butterfly plot) or nanodiscs (bottom butterfly plot). Each plot compares the deuterium uptake profile for the apo P-gp with the vanadate-trapped state and percent deuterium uptake at each time point is plotted at the midpoint of each peptide sequence. Solid connecting lines indicate continuous coverage, whereas dotted lines reflect missing sequence coverage. B, difference deuterium exchange profile of P-gp peptides in apo P-gp and vanadate-trapped P-gp (apo minus vi-trapped) at varying times of exposure to D₂O. Dotted line represents the sum of differences between the two conditions across all time points, and thus the cumulative difference in deuterium exchange. Positive values or negative values indicate regions of P-gp that become more or less protected in the vanadate-trapped state, respectively.

C, difference in solvent accessibility between the apo and Vi-trapped states for P-gp detergent micelles at 1 h, mapped onto P-gp structures in the inward- and outward-facing conformations (IF and OF; PDB code 49QH (murine P-gp) and PMDB code PM0075213 (human P-gp based on SAV1866 OF conformation)). Modeler was used to fill in missing residues (such as the linker) in the PDB files to display H/DX information. Regions in P-gp that become moderately exposed (cyan, 0 to ≤ 5%), moderately protected (yellow, 0 – 5%), highly protected (orange and red, > 5% and > 10%) in the vanadate-trapped state relative to the apo state are shown. Structures are rotated 180° and shown in the lower panel. D, same as C but for P-gp nanodiscs. D-H, D and H loops.
Figure 4. Peptides displaying EX1-related kinetics in ligand-free P-gp in lipid/detergent micelles or nanodiscs. A, representative isotopic mass envelopes of peptides (residues labeled at top of the panels) with bimodal kinetics in nanodiscs (right) but not in lipid/detergent micelles (left). The centroid of the low and high mass distributions was used to plot the deuterium uptake for the two populations (low mass, solid line; high mass, dashed line) (bottom graph in each panel, data from nanodiscs). Rate of relaxation is measured from the rate of disappearance of the low mass population and peptides are categorized into fast (≤ 1 min, red), moderate (> 1 min ≤ 4 h, yellow), and slow (> 4 h, purple) (refer to supporting Fig. S2 for details). B, comparison of the localization of bimodal peptides across the linear sequence for P-gp in lipid/detergent micelles or nanodiscs. Peptides are colored according to relaxation half-lives; fast, red; moderate, yellow, and slow, purple. Regions colored gray did not show EX1-like behavior. Lines indicate pairs of distant peptides based on the primary sequence of P-gp that are in close proximity (or potentially close) in the tertiary structure. C and D, location of these peptides plotted onto the IF conformation of P-gp for lipid/detergent micelles or nanodiscs, respectively.
peptides undergo conformational transitions over the course of a minute, whereas others take several minutes to a few hours. Interestingly, some peptides that are far apart based on the primary sequence but interact closely with each other in the tertiary structure of the protein exhibit EX1 kinetics on a similar time scale, such as ICL1-(153–167) and ICL4-(901–915), which extend as coupling helices to NBD 1 (Fig. 3D). Peptides with slower relaxation rates are found at both of the NBDs in the ATP-binding sites. These peptides are mapped onto the structure of P-gp in Fig. 3, C and D, and some are discussed in greater detail below.

To ensure that the unusually slow conformational exchange revealed by the bimodal EX1 kinetics of some peptides was not simply due to time-dependent denaturation of P-gp, we monitored the functional properties of P-gp in detergent and nanodiscs during the time course used to monitor H/DX. P-gp in nanodiscs, and separately in detergent/lipid micelles, was preincubated at 25 °C for varying durations and then assayed for ATPase activity by a standard colorimetric method (see “Experimental procedures”). Verapamil was added postincubation to the ATPase assay buffer to produce an appreciable increase in phosphate release. The results are shown in Fig. S3A. The stimulated ATPase activities of P-gp in detergent and nanodiscs are constant throughout the entire course of the 4-h H/DX experiment. As an additional probe of the functional effects of the time-dependent changes in conformation observed by H/DX, we preincubated a sample of P-gp nanodiscs for 4 h at 25 °C prior to H/D exchange. After 4 h we performed an H/D exchange experiment and monitored deuterium uptake after 1 min. The results were essentially identical to the deuterium uptake observed at 1 min without preincubation (Fig. S3B). This result demonstrates that the slow conformational exchange observed in the EX1 kinetic behavior is not the result of an irreversible, nonequilibrium, change such as degradation or aggregation. Rather the slow EX1 kinetics reflect native conformational dynamics of equilibrating states.

**Effect of vanadate trapping on EX1 kinetics**

As noted above, several peptides exhibit EX1 kinetics on varying time scales (Fig. 4). Additional examples are shown in the supporting information (Fig. S4), and include peptide-(494–508), peptide-(593–611), and peptide-(1036–1053). Vanadate trapping slows down the rates of EX1 transitions for some, but not all, of these peptides. Two peptides with “moderate” and “fast” EX1 kinetics that are sensitive to Vi trapping, respectively, are peptide-(901–915) and peptide-(1036–1053) (Fig. 5). For peptide-(1036–1053) that is located in an unstructured loop region after TM12 but prior to WA (NBD2), the increase in $t_{1/2}$ for conformational exchange upon Vi trapping is estimated to be ~12-fold, from 47 s to 9.6 min (supporting Fig. S4). This is consistent with the general “tightening” of the NBDs noted in Fig. 2, with some peptides more affected than others. For peptides that exhibit slower EX1 kinetics, such as peptide-(901–915), the rate constant for exchange in the presence of ATP and Vi could not be measured accurately within the resolution of these experiments due to a small number of time points, although the effect on EX1 rates is directly observable. This effect of vanadate trapping on the rate of conformational exchange, reflected in the EX1 kinetics, is presumably coupled
to the shift toward the OF conformation and increased NBD1–NBD2 interactions.

Discussion

The results provide an experimentally-based measurement of the conformational dynamics for specific peptides of P-gp in a lipid bilayer and they reveal several features of nucleotide-dependent, and environmentally sensitive, P-gp dynamics. The results further provide new information about the energy landscape of the IF and OF conformational ensembles and their interconversion in a lipid bilayer.

The first important observation is that the global dynamics of P-gp are qualitatively very similar in lipid/detergent micelles versus in lipid nanodiscs, including both ligand-free and “vana-date-trapped” states. This provides assurance that the structure of solubilized P-gp in the presence of lipid faithfully mimics the overall structure in the lipid bilayer. There are, however, modest differences between the H/DX behavior in the two environments, which may be critically important for interpreting some results in the literature. Because the behavior of P-gp in the two environments is qualitatively similar, the discussion highlights important conclusions that span both environments. First the ligand-free P-gp is considered followed by the Vi-trapped state.

The most striking result is the large number of peptides in the ligand-free IF state that exhibit EX1 kinetics. Generally, all of the peptides that display bimodal EX1 kinetics are found in connecting loop regions between β strands or α-helices in the NBDs or ICLs. This is consistent with MD simulations where local fluctuations in these regions contribute to global, conformational dynamics of P-gp (12, 17).

Of particular importance is the wide range of time scales for the EX1 kinetics. Conservatively, at least in two time regimes are evident, as indicated in Fig. 4. This requires that there are at least two energy barriers (transition states) for the conformational exchange taking place in the nucleotide-free P-gp, and likely more, if only a single transition state existed, for example, a simple IF to OF transition, then all peptides with EX1 exchange behavior would exhibit a similar time scale for the conformational exchange. Therefore, the presence of multiple transition states for conformational exchange in the nucleotide-free state demands that there are multiple conformations and the simple two-state IF to OF model is oversimplified. Even if one of the observed time regimes for EX1 kinetics, and hence one energy barrier, corresponds to the overall IF to OF exchange, then at least one additional energy barrier must exist to yield EX1 kinetics with distinct time scales. As noted above, recent data based on cryo-EM (10), FRET (11), and DEER (12) indicate an ensemble of IF states with varying degrees of NBD separation; however, no information has been available about the time scales of transitions between the states. The H/DX data support and extend those studies. There must be more than one conformation contributing to the ligand-free conformational ensemble, and at least some of them exchange on remarkably slow times scales. In effect, there is a “rough” conformational landscape for the ensemble of NBDs in nucleotide-free P-gp, with energy barriers of varying magnitude.

To the extent that the conformational heterogeneity of the NBDs is transmitted to the TMHs, the potential functional significance of the rough landscape of the ligand-free P-gp is obvious: the presentation of multiple persistent conformations to drug substrates in the membrane makes possible a conformational selection mechanism for achieving the extreme substrate promiscuity of P-gp. This does not exclude any possible contribution of induced fit, and both may occur (25, 26). Although induced fit has been suggested to be a source of promiscuity with P-gp (15, 27), the likely contribution of conformational selection seems to have been ignored. In fact, the combination of induced fit and conformational selection may maximize substrate promiscuity of detoxication enzymes and transporters (28).

The existence of such conformational heterogeneity in the Vi-trapped state is unexpected, in as much as “transition states” are usually considered to include high frequency local dynamics but not larger amplitude slow movements. The lifetimes of reaction transition states are too short to be coupled to slow conformational changes, and the conformational changes observed here in the trapped state are not coupled to catalysis. Clearly, the well-established rates of ATP hydrolysis by P-gp are much faster than the conformational exchange of some peptides in the Vi-trapped state. The very different time scales for some conformational exchange observed here and the rates of ATP hydrolysis suggest interesting possibilities. Steady state basal and substrate-stimulated ATPase activities are ensemble-averaged rates. It is possible that different conformations have very different k_{cat} values for hydrolysis. It should be stressed that the average would be dominated by the conformations with the fastest k_{cat} values. The ensemble is not conformationally synchronized so, at any time, the k_{cat} reflects the conformationally averaged properties and some conformations may not participate in catalysis until they rearrange, whereas other conformations contribute to fast catalysis. Alternatively, the different conformations that contribute to the ensemble at any moment may all have similar k_{cat} values for ATP hydrolysis. It should be stressed that the slow conformational changes observed here do not necessarily correlate with large amplitude changes. For example, the fraying of the end of a helix remote from the transition state may not cause any change in k_{cat} for hydrolysis, although it leads to a slow shift to a state with rapid H/D exchange. Conformational heterogeneity does not necessitate catalytic heterogeneity. Furthermore, due to high concentrations of ATP in vivo, the OF ensemble is expected to represent a large fraction of the P-gp, even in the absence of drugs. Thus, the conformational heterogeneity of the OF ensemble with nucleotide bound would allow conformational selection and further contribute to substrate promiscuity. Of course, an additional consideration is the nature of the Vi-trapped state, which is only a model for the hydrolytic transition state and may artificially restrict conformational motion. However, the widespread EX1 kinetics observed for the substrate-free P-gp suggest that the conformational heterogeneity observed in the trapped state is not completely artificial.

Interestingly, the number of P-gp peptides that exhibit EX1 kinetics in detergent is less than the number that exhibit EX1 kinetics in the nanodiscs, suggesting a broader range of persistent conformations in the more native membrane. Speculatively, evolution has optimized the rough landscape of P-gp in...
the membrane to fully exploit the conformational selection and substrate promiscuity for drugs that partition into it.

Vanadate trapping has a wide range of effects on the EX1 kinetics of different peptides, but in the nanodiscs and detergent solution, the effect of ATP and vanadate on the overall H/DX profile is modest. Most models for P-gp dynamics include intimate contact between NB1D and NB2D upon formation of the OF state, whereas they are well-separated and apparently more solvent-exposed in the IF state. In fact the NBDs thermodynamically stabilize one another in the vanadate-trapped state (29), which implies substantial inter-NBD interaction, as suggested by the effect of vanadate trapping on EX2 kinetics for many peptides spanning the NBDs. Thus, the combined H/DX results indicate that, either the expected contact between the NBDs upon vanadate trapping of the OF state only modestly affects solvent access to most structural elements including those that are stabilized by NBD dimer formation, or the NBDs remain in a dynamic equilibrium between states that allow solvent access even when the ATPase activity is persistently inhibited and the NBDs form a dimer. Notably, there is no crystal structure of the OF state of P-gp so it is difficult to assess the solvent accessibility of the NBD–NBD interface. The cryo-EM studies in detergent micelles indicate that the vanadate-trapped state is completely OF with some interactions between NBDs. The resolution of those structures, however, does not allow for detailed analysis of the solvent accessibility of the NBD–NBD interface. In contrast, the elegant DEER studies by Verhalen et al. (30) indicate that most of the population shifts in the presence of verapamil, vanadate, and ATP so that the NBDs are in close proximity, but residual components of the population with partially separated NBDs remain. Those studies were also done in micelles, and did not include the state analogous to our vanadate-trapped state.

It is interesting that the ATP–vanadate-induced decrease in HD/X in our studies is slightly greater for P-gp in detergent than in nanodiscs. A similar observation has been reported for BmR in detergent versus membrane based on H/DX (31). This suggests that closure of the NBDs to the OF ensemble is more complete in detergent or the IF state retains some NBD–NBD interaction in the nanodiscs, or both. MD simulations have also shown that the conformations of P-gp in detergent deviate from P-gp in a membrane environment (32).

Last, it is striking that extremely slow conformational changes coupled to ATPase activity have been observed for the ABC transporter BtuC2D2 (33). The hydrolysis of ATP was improved with increasing turnover over the course of days. Possibly, the slow conversion to the most active conformations is related to the intrinsic slow conformational changes observed here for P-gp, but additional work is needed to relate these observations.

In summary, these data provide the first details of the energy landscape of ligandfree P-gp in a lipid bilayer. This landscape includes a minimum of three states, and based on the wide range of time scales of the EX1 kinetics for multiple peptides, the landscape is likely more complex. The results are schematized in Fig. 6, and suggest the likely contribution of conformational selection to the substrate promiscuity of P-gp.

**Experimental procedures**

**P-gp and MSP1D1 expression and purification**

Hexa-histidine-tagged MSP1D1 was expressed in *Escherichia coli* and purified as described (34). Expression of Histagged P-gp in *Pichia pastoris* and purification were carried out as described (35).

**Reconstitution of P-gp in detergent/lipid micelles or nanodiscs**

1 mg/ml of purified P-gp was solubilized in buffer containing 20 mM Tris, 100 mM NaCl, 1 mM TCEP, 0.1% dodecyl maltoside, pH 7.4. DMPC lipid was added to a final lipid/protein ratio of 2:1 (w/w) for activation of P-gp, and for optimal micelle formation with a lipid/detergent ratio (or R_{soy}) of 2 (w/w) (36, 37). Reconstitution of P-gp into DMPC nanodiscs was carried out as described (35). P-gp nanodiscs were separated from empty nanodiscs by SEC-HPLC chromatography (Superdex 200 10/300 GL column, GE Healthcare) and concentrated up to 1 μm using a 100-kDa MWCO spin filter (Millipore).

**Hydrogen/deuterium exchange**

P-gp micelles or nanodiscs were incubated in the absence or presence of 10 mM MgATP and 2.4 mM vanadate for 15 min at 25 °C, prior to hydrogen-deuterium exchange experiments. Exchange reactions for P-gp nanodiscs were initiated by a 10-fold dilution into deuterated buffer containing 95% D₂O.
were trapped and desalted on a trap C8 column flowing 0.1% D2O. The final mixture was filtered using a cold microcentrifuge spin filter (0.45-μm cellulose acetate) at 4°C for 30 s, before rapidly frozen in liquid nitrogen and stored at −80°C until analysis. All reactions contained the tetrapeptide, PPPI, as an internal standard to ensure consistency in mass spectrometry analyses across samples. Undeuterated samples were prepared with the steps described above, except that Optima LC-MS grade H2O was used in place of D2O.

**Mass spectrometry**

Samples were thawed on ice for 5 min and injected onto a refrigerated Waters nanoAcquity H/DX-UPLC system coupled to a Synapt G2-S QTOF mass spectrometer (Waters). Peptides were trapped and desalted on a trap C8 column flowing 0.1% formic acid (FA) with 0.02% trifluoroacetic acid (TFA) at 200 μl/min for 3 min (ACUITY UPLC BEH C8 1.7 μm VanGuard column, Waters), followed by separation using an analytical C8 column (ACUITY UPLC BEH C8 1.7 μm, 1×100 mm column, Waters) running a gradient of 5–40% solvent B for 8 min (solvent A, 0.1% FA, 0.02% TFA, and 5% acetonitrile (ACN); solvent B, 0.1% FA, and 80% ACN) at 40 μl/min. At the end of UPLC gradient, sample flow from the column was diverted to waste to prevent excess cholate from entering the mass spectrometer. To minimize sample carryover, the analytical column was cleaned with repeated fast gradients of 10–100% solvent B, whereas the syringe, loop, and trap column were washed with a series of 10% FA, 50% trifluoroethanol, 80% methanol, 2:1 isopropanol alcohol/ACN, and 80% ACN (38). The electrospray ionization source was operated in the positive ion mode and ion mobility was enabled for the instrument. Peptide identification was done by tandem MS/MS using a combination of data independent acquisition methods with ion mobility (High Definition MS3) and data-dependent MS/MS acquisition. Peptidic fragments of P-gp were identified through database searching in ProteinLynx Global Server version 3 (Waters) as well as through Protein Prospector. Relative deuterium uptake based on the centroid of isotopic distribution was processed by DynamX version 3 (Waters) and deuterium incorporation was not corrected for back-exchange. As is customary with deuterium uptake plots, no error bars are included. The precision of the measurements is extremely high with typical errors for each peptide in the mass range analyzed here of ±0.2 atomic mass unit. A threshold of 1.5% difference in deuterium content between species was used to evaluate significant changes in deuteration kinetics between protein states based on previous analyses of variation in H/DX data (39, 40). Each deuterium uptake plot is representative of three separate experiments with different P-gp nanodisc preparations. Bimodal mass spectra were deconvoluted using HX-Express 2 to extract fractional contribution by individual mass envelopes. The rate of relaxation was determined by fitting the fractional decay of the lower mass species with a first-order exponential.

**Activity assay**

The hydrolysis of ATP by P-gp (ATPase activity) was determined from the amount of Pi released from ATP hydrolysis, based on a colorimetric method adapted from Chifflet (41). P-gp in nanodiscs (1 μg) or detergent/lipid micelles (3 μg) were aliquoted into tubes and incubated at 25°C for varying durations (0 to 4 h). At the end of incubation, assay buffer (50 mM Tris, 150 mM NH4Cl, 5 mM MgCl2, 0.02% NaN3, 10 mM TCEP, 100 μM verapamil, 1 mM ATP, pH 7.4) in the absence or presence of 240 mM Vi was added to initiate the reaction. After 30 min at 37°C, the reaction was stopped by adding EDTA to a final concentration of 10 mM. The absorbance intensity from the formation of phosphomolybdate was measured at 850 nm using a Tecan Infinite M200 microplate reader.

**Loop modeling of missing regions in P-gp structures**

H/DX results were superimposed on available crystal structure of IF P-gp, Protein Data Bank (PDB) code 4Q9H, and OF model of P-gp, Protein Model Database 0075213, and these represent various possible conformations of P-gp. Missing residues were added as loop regions using Modeler 9.18 with standard loop modeling protocol (42). For the IF structure, residues in the linker region, peptide-(627–688), were filled but not the N-terminal residues, 1–29, because these were not observed in our H/DX experiments. Similarly, linker residues, peptide-(630–696), were added for the OF structure, including missing residues in extracellular loop 1, peptide-(82–101), but the N-terminal residues 1–35 were omitted. Fifty models were generated and models were selected based on scoring criteria from the results of running Modeler from Chimera 1.11.2, and assessed for steric clashes with default clash criteria. The models served the primary purpose of displaying H/DX data and were not intended as precise prediction of P-gp conformations.

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**Conformational dynamics of P-gp**

(Cambridge Isotope Labs), 20 mM Tris, 100 mM NaCl, 2 mM MgCl, pH 7.4, and samples were incubated at 25°C for various times. Deuterated buffer including 0.1% dodecyl maltoside was used for exchange reactions with P-gp micelles. Samples were quenched with an equal volume of ice-cold quench buffer (300 mM potassium P2, 4 mM guanidine hydrochloride, 250 mM TCEP (tris(carboxyethyl)phosphine), pH 2.5), followed by an addition of 25:1 sodium cholate/DMPC, and the entire mixture was transferred rapidly to ice-cold immobilized pepsin (Pierce) for a 5-min digestion. At the last minute of digestion, 3 μg of ZrO2-coated silica resin (Hybrid SPE resin, Sigma) (or 10 μg for P-gp micelles) was added to the mixture and incubated on ice for the remaining duration to remove excess lipid. The final mixture was filtered using a cold microcentrifuge spin filter (0.45-μm cellulose acetate) at 4°C for 30 s, before rapidly frozen in liquid nitrogen and stored at −80°C until analysis. All reactions contained the tetrapeptide, PPPI, as an internal standard to ensure consistency in exchange conditions across samples. Undeuterated samples were prepared with the steps described above, except that Optima LC-MS grade H2O was used in place of D2O.
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