Transmision State Analysis of the Coupling of Drug Transport to ATP Hydrolysis by P-glycoprotein

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ATPase activity associated with P-glycoprotein (Pgp) is characterized by three drug-dependent phases: basal (no drug), drug-activated, and drug-inhibited. To understand the communication between drug-binding sites and ATP hydrolytic sites, we performed steady-state thermodynamic analyses of ATP hydrolysis in the presence and absence of transport substrates. We used purified human Pgp (ABC1, MDR1) expressed in Saccharomyces cerevisiae (Figler, R. A., Omote, H., Nakamoto, R. K., and Al-Shawi, M. K. (2000) Arch. Biochem. Biophys. 376, 34–46) as well as Chinese hamster Pgp (PGP1). Between 23 and 35 °C, we obtained linear Arrhenius relationships for the turnover rate of hydrolysis of saturating MgATP in the presence of saturating drug concentrations (kcat), from which we calculated the intrinsic enthalpic, entropic, and free energy terms for the rate-limiting transition states. Linearity of the Arrhenius plots indicated that the same rate-limiting step was being measured over the temperature range employed. Using linear free energy analysis, two distinct transition states were found: one associated with uncoupled basal activity and the other with coupled drug transport activity. We concluded that basal ATPase activity associated with Pgp is not a consequence of transport of an endogenous lipid or other endogenous substrates. Rather, it is an intrinsic mechanistic property of the enzyme. We also found that rapidly transported substrates bound tighter to the transition state and required fewer conformational alterations by the enzyme to achieve the coupling transition state. The overall rate-limiting step of Pgp during transport is a carrier reorientation step. Furthermore, Pgp is optimized to transport drugs out of cells at high rates at the expense of coupling efficiency. The drug inhibition phase was associated with low affinity drug-binding sites. These results are consistent with an expanded version of the alternating catalytic site drug transport model (Senior, A. E., Al-Shawi, M. K., and Urbatsch, I. L. (1995) FEBS Lett. 377, 285–289). A new kinetic model of drug transport is presented.

Human P-glycoprotein (Pgp), which interacts with a structurally diverse set of cytotoxic agents, is a very broad specificity drug pump, located at the plasma membrane of cells. Due to its central role in compromising cancer chemotherapy and in modulating the bioavailability and distribution of therapeutic agents, Pgp is of great clinical importance (1, 2). Pgp is a member of the ATP-binding cassette superfamily of transport proteins (3–5). It consists of two homologous domains; each domain has six transmembrane helices and an ATP site. Additionally, each domain is thought to have a structure similar to the solved x-ray structure of the homologous Escherichia coli MsrA lipid A transporter monomer (6). Photolabeling and genetic studies have indicated that drug-binding sites are located in the transmembrane region (reviewed in Ref 1). Binding of drugs stimulates ATP hydrolysis at the two nucleotide sites in an alternating fashion, leading to drug transport (7). One confounding factor in the analysis of drug transport by Pgp is the presence of basal ATPase in the absence of any added transport substrates (8, 9). This has usually been ascribed to the presence of an unidentified endogenous transport substrate or to lipids being flipped in purified preparations (10–12). Recently, cholesterol was proposed as the transport substrate leading to basal ATPase (13). Alternative explanations have also been proposed (14). In this study, we investigated the mechanistic origin of this basal ATPase activity.

In Pgp, conformational changes mediate the coupling of energy derived from the hydrolysis of ATP to the transmembrane movement of drug (12, 15–20). To probe this coupling of drug transport to ATP hydrolysis, we measured the thermodynamics of the overall rate-limiting transition state, which appeared to be related to a conformational change step. This analysis was performed as a function of ATP and transport drugs using purified yeast-expressed human Pgp (21) and Chinese hamster Pgp (22). The availability of these transport–competent (23), homogeneous, and high specific activity preparations allows this analysis to be performed. Previously, we had developed the thermodynamic methods employed here to study rotational coupling of ATP synthesis/hydrolysis to the movement of protons in the F0F1-ATP synthase (reviewed in Ref. 24). In this study, we present a detailed thermodynamic description of the coupling transition state and use this information to reframe our model of drug transport by Pgp (7).

EXPERIMENTAL PROCEDURES

Sources of Pgp Used—C-terminally His6-tagged wild-type human Pgp (ABC1, MDR1 gene product) was expressed in Saccharomyces cerevisiae plasma membranes in the presence of 10% glycerol as a chemical chaperone from the yeast expression plasmid YEpMDR1HIS. Microsomes were prepared, and Pgp was solubilized and purified to homogeneity as described previously (21). Proteoliposomes were made by dilyis as described previously (9) and stored frozen at -80 °C in 50 mM Tris-Cl (pH 7.4), 1 mM dithiothreitol, 1 mM EGTA, and 5 mM 6-aminohexanoic acid.

Chinese hamster ovary Pgp (pgp1 gene product) was expressed in Chinese hamster ovary CR1R12 cells grown in suspension culture. Plasma membranes were prepared from the harvested cells as de-
scribed previously (8). The membranes were solubilized, and Pgp was purified as described previously (22). Proteoliposomes were made as described above.

Lipid stocks were prepared as described previously (21) and stored at −80 °C under argon. In this study, two lipid preparations were used: E. coli ether/acetone-precipitated lipids (E. coli lipids) and a mixture of lipids (mixed lipids) composed of 60% (w/w) E. coli lipids, 15.7% egg phosphatidylcholine, 10% bovine brain phosphatidylethanolamine, and 12.5% cholesterol.

ATPase Assays—Standard ATPase assays were performed in 40 mM Tris/HSO4 (pH 7.4), 10 mM NaATP, 15 mM MgSO4, 0.1 mM EDTA, 2 mM NaN3, and 1% (v/v) MeSO4 at 37 °C in the presence and absence of transport drugs as indicated. Samples (50 μl) were removed at appropriate times (four time points to ensure linearity) and injected into 1 ml of ice-cold 8 mM EDTA (pH 8) to stop the reaction (25). Liberated P was determined by the method of Van Veldhoven and Mannaaerts (26) as modified by Al-Shawi et al. (27).

Drug Titrations—Drug titrations of ATPase activities were fitted to two steady-state kinetic models. The first is a non-partitioning model (Model 1) composed of basal ATPase activity (uncoupled activity in the absence of drugs), drug-activated ATPase activity (at activating drug concentrations), and drug-inhibited ATPase activity (at high inhibitory concentrations of drug). In this model, basal and drug-stimulated activities are independent entities and do not influence each other, but each is inhibited by inhibitory concentrations of drug. The kinetic equation is given by Equation 1,

\[ v = (B + (D \cdot [drug])/(K_{s\text{drug}} + [drug])) \cdot (1 - ([drug]/K + [drug])) \]  

(Eq. 1)

where \( v \) is the ATPase activity, \([\text{drug}]\) is the drug concentration, \( B \) is the basal ATPase activity, \( D \) is the maximal ATPase activity associated with drug activation, \( K_{s\text{drug}} \) is the apparent Michaelis constant for drug activation, a virtual apparent dissociation constant of drugs from all drug-bound Pgp species to water, and \( K \) is the inhibition constant for drug inhibition. Kinetic equations similar to Equation 1 have been used previously to fit the drug-dependent ATPase activity of Pgp (28, 29).

Model 1 could conceivably arise in the presence of a tightly bound noncompetitive endogenous substrate or by a permanent activation of basal ATPase of Pgp by a covalent modification such as phosphorylation. The second steady-state kinetic model is an activity-partitioning model (Model 2) (23) in which basal and drug-stimulated activities are dependent entities, and Pgp preparations are independent. The ATPase activities were determined by the non-parametric Mann-Whitney rank sum test. The average molecular mass of olive oil was calculated to be 867 Da with an average density of 0.912 g/ml at 25 °C (79).

Routine Methods and Materials—SDS-PAGE and Western blot analysis were carried out as described previously (8). Protein concentrations were determined using Amido Black 10B according to the method of Kaplan and Pedersen (31). Concentrations of ionic species of magnesium and ATP were calculated using the algorithm of Fabiato and Fabiato (32). General reagents and chemicals were purchased from Aldrich or Sigma. Phosphoenolpyruvate and pyruvate kinase were from Roche Applied Science. Detergents were from Calbiochem and Incolo Pharmaceuticals. Lyso phosphatidylcholine and lipids were from Avanti Polar Lipids, except bovine brain phosphatidylethanolamine was from Sigma.

Statistics—Statistical analyses were performed using SigmaStat 2.0 (Jandel Scientific). Linear trends were compared by fitting each population of experimental points to be tested to a linear function by least-squares regression analysis. Using the resultant regression coefficients (gradient and intercept), apparent gradient and intercept values were calculated for each experimental point. The numerical product of the apparent gradient multiplied by the apparent intercept for each experimental point was then obtained. To calculate the probability of two lines being significantly different, populations of products were then tested against each other by the non-parametric Mann-Whitney rank sum test.

Data available at www.internationaloilseedoil.org.

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S. A. Aravamudhan et al., "Steady-state Thermodynamics of P-glycoprotein," Biochemistry, vol. 38, no. 48, pp. 15195-15204, 1999.
RESULTS

Tight Coupling of ATP-binding and Drug Transport Sites—Using drug transport–competent proteoliposomes, Pgp ATPase activities were determined as a function of drug concentration. Fig. 1 clearly shows that there was communication between the nucleotide- and drug-binding sites. The ATPase activity at the two nucleotide sites was strongly controlled by the concentration of drug present. The lines drawn are kinetic fits (Equation 2) to the data generated using our transport model as described previously (23). Fig. 1 reiterates our previous findings and illustrates that drug control of ATPase activity in transport–competent proteoliposomes is characterized by three distinct phases: (i) basal ATPase in the absence of drug (transport substrate), (ii) a single $K_{M(0)}$ for activation of ATP hydrolysis at both nucleotide sites associated with inward facing (cellular orientation) high affinity drug-binding sites (see Refs. 23 and 33), and (iii) a single $K_i$ for inactivation of ATP hydrolysis associated with the saturation of outward facing low affinity drug-binding sites (demonstrated in Ref. 23). In the case of valinomycin, only the basal ATPase and drug activation phases were seen (Fig. 1). This is thought to be due to the limited solubility, high lipophilicity, and high membrane permeability (34) of valinomycin such that an inhibitory concentration to saturate the outward facing low affinity drug-binding sites cannot be technically achieved (see also Ref. 35).

Resolutions of the Three Phases of Drug-dependent ATPase Activity by Thermodynamic Analyses—To study the interaction of Pgp with drugs during transport, we performed Arrhenius analysis of ATPase activity as a function of drug concentration. Transport drugs analyzed included verapamil, spin-labeled verapamil (SL-verapamil) (23), colchicine, valinomycin, rhodamine 123, Hoechst 33342, and vinblastine at the concentrations detailed under “Experimental Procedures.” Drugs were chosen as representative members of the major classes interacting with drug transport sites. Shapiro and Ling (36) first demonstrated the existence of two cooperative drug-binding transport sites, which they termed the “H-site” and “R-site.” Hoechst 33342, colchicine, and SL-verapamil all interact with the H-site. Rhodamine 123 is the archetypal R-site drug. Vinblastine and verapamil have been found to interact with both types of sites (36, 37). Valinomycin was found to interact with a “circular peptide site,” which also interacted with verapamil (38). Clearly, the circular peptide site also has overlapping specificities with the drug-binding H- and R-sites (36, 37). Ligands binding exclusively to the “modulator site” (39, 40) and “steroid-binding sites” (37, 41, 42) were not used in this study.

Linear Arrhenius relationships were obtained between 23 and 35 °C for ATP turnover and drug transport by both Chinese hamster ovary Pgp and human Pgp. Both E. coli lipid and mixed lipid proteoliposomes were used in this study (examples are shown in Fig. 2A). The linearity of the plots indicated that single overall rate-limiting steps were being observed. In contrast, some plasma membrane preparations and Pgp reconstituted in synthetic lipid mixtures did not produce linear Arrhenius relationships due to lipid phase transitions and other effects (43–45). Arrhenius relationships were measured for the drugs listed above as a function of concentration such that all phases of drug-dependent ATPase activities were investigated. It should also be noted that Pgp activity at 37 °C represents a mixed component “optimal activity.” This optimal activity contains the activation component (as measured in this study) and a reversible temperature inactivation component.4

In previous Arrhenius analyses of ATPase activity of Pgp, the turnover numbers were not accurately known. Hence, it was possible to determine only the apparent enthalpic activation energy of the rate-limiting transition state (46–48). In this study, we used fully activated pure P-glycoproteins reconstituted under controlled conditions, which allowed us to calculate the true turnover numbers for any given assay condition. Employing standard thermodynamic equations (see “Experimental Procedures”) (30), we calculated the enthalpic, entropic, and free energy terms for the rate-limiting transition states. Initially, apparent thermodynamic parameters were calculated for the three phases of ATPase activity, viz. basal activity, drug activation, and drug inhibition. Examples of these calculated values are given in Table I. These apparent values are only relevant to the exact conditions of the assays employed and are not predictive by themselves. True thermodynamic trends cannot be easily ascertained in the raw primary data. However, from these primary data, the intrinsic thermodynamic parameters of drug transport could be calculated as described below.

The apparent activation energies ($E_a$) of basal ATPase for MDR1 in mixed and E. coli lipids were 104.2 ± 2.9 and 117.9 ± 6.6 kJ/mol, respectively. The analogous values for PGP1 were 115.3 ± 6.6 and 87.0 ± 2.5 kJ/mol, respectively. Different $E_a$ values obtained with different lipids indicate the importance of lipids in controlling the ATPase activity (47, 49, 50). The relatively large $E_a$ values indicate that the rate-limiting step of basal ATPase is characterized by large conformational rearrangements. For comparison, the activation energies for ATP-driven work by the E. coli F$_0$F$_1$-ATP synthase (51), the guinea pig kidney Na$^+$,K$^+$-ATPase (52), and the rabbit muscle myosin ATPase (53) were only 34, 71, and 70 kJ/mol, respectively. Large $E_a$ values were also seen in the presence of drug substrates (Table I), again indicating that large conformational rearrangements occur in the rate-limiting steps during drug activation and drug inhibition of ATPase activity. An activation energy of 134 kJ/mol was observed for the nucleotide transport by the beef heart mitochondrial ADP/ATP transporter (54). In these studies.

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3 H. Omote and M. K. Al-Shawi, unpublished data.
4 M. K. Al-Shawi, unpublished data.
it was concluded that the “remarkably high activation energy” of the translocation step was the result of large conformational changes by the carrier and changes in its interactions with membrane phospholipids. The activation energy for the Pgp ATPase reaction using activating drug concentrations was the same as that for drug transport. This is significant. For example, when 50 μM SL-verapamil was transported by human Pgp into mixed lipid proteoliposomes, the \( E_a \) values for the ATPase and transport reactions (as measured by EPR spectroscopy) (23) were 101.1 ± 11.0 and 102.8 ± 11.2 kJ/mol, respectively. Thus, both reactions were coupled and limited by the same coupling conformational changes.

### Relationship between the Three Phases of ATPase Activity

Thermodynamic tests of the inter-relationships between the three phases of ATPase activity and verification of the identities of the rate-limiting transition states were performed by the methods of Exner (55). For any single rate-limiting transition state, there is a linear free energy relationship between two rate constants for the reaction \( (k_1 \) and \( k_2 \) measured at two temperatures \( T_1 < T_2 \), which is given by Equation 8,

\[
\log k_2 = a + b \log k_1
\]

(Eq. 8)

where \( a \) and \( b \) are constants. Equation 8 can be used to distin-

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**Table I**

| Enzyme* | Transport drug at given conc | Drug activity phase | \( E_a \) (kJ/mol) | \( ΔH^\circ \) (kJ/mol) | \( TΔS^\circ \) (kJ/mol) | \( ΔG^\circ \) (kJ/mol) | Experiments averaged |
|----------|-------------------------------|--------------------|--------------------|------------------------|------------------------|------------------------|---------------------|
| Human    | None                          |Basal               |104.2 ± 2.9        |27.4 ± 2.9             |74.2 ± 0.1             |32                     |
| Human    | 130 μM verapamil              |Activation†         |99.3 ± 7.6         |26.1 ± 7.7             |70.6 ± 0.2             |16                     |
| Human    | 400 μM verapamil              |Inhibition‡         |94.9 ± 9.7         |21.5 ± 9.5             |70.8 ± 0.2             |2                      |
| Human    | 50 μM SL-verapamil            |Activation          |101.1 ± 11.0       |27.3 ± 11.4            |70.2 ± 0.4             |2                      |
| Human    | 130 μM vinblastine            |Activation          |112.0 ± 7.9        |37.7 ± 8.0             |71.7 ± 0.2             |5                      |
| Human    | 2 mM colchicine               |Activation          |122.3 ± 3.6        |45.2 ± 3.9             |74.5 ± 0.5             |4                      |
| Human    | 15 mM colchicine              |Inhibition          |115.7 ± 7.0        |38.8 ± 7.8             |74.4 ± 0.8             |2                      |
| Human    | 0.4 mM rhodamine 123          |Activation          |116.2 ± 6.0        |40.9 ± 6.1             |72.8 ± 0.1             |2                      |
| Human    | 55 μM Hoechst 33342           |Activation          |95.3 ± 10.5        |17.5 ± 11.2            |75.2 ± 0.7             |2                      |
| Human    | 2 μM vinblastine              |Activation          |95.5 ± 7.5         |20.2 ± 8.0             |72.7 ± 0.5             |2                      |
| Human    | 40 μM vinblastine             |Inhibition          |84.6 ± 10.2        |6.8 ± 11.0             |75.2 ± 0.8             |2                      |
| Hamster  | None                          |Basal               |87.0 ± 2.5         |12.2 ± 2.5             |72.2 ± 0.1             |11                     |
| Hamster  | 50 μM verapamil               |Activation          |110.2 ± 3.1        |38.4 ± 2.7             |69.2 ± 0.5             |4                      |
| Hamster  | 130 μM verapamil              |Inhibition          |106.0 ± 9.9        |33.9 ± 9.9             |69.5 ± 0.1             |2                      |
| Hamster  | 130 μM vinblastine            |Activation          |103.5 ± 3.8        |33.4 ± 3.4             |67.6 ± 0.2             |12                     |

* Purified human Pgp (MDR1) was reconstituted into mixed lipid proteoliposomes.
† Activating drug concentrations were optimal, leading to maximal activation of ATPase activity.
‡ Inhibiting drug concentrations were concentrations of drug that inhibited ATPase activity for drugs with an ATPase-activating component.
* Purified Chinese hamster Pgp (PGP1) was reconstituted into E. coli lipid proteoliposomes.
guish between transition states of different reactions because reactions rate-limited by the same transition state would fall on the same line when \( \log k_2 \) is plotted as a function of \( \log k_1 \). To test the Pgp data for transition state relationships, all log activities at 35 °C were plotted against the corresponding log activities at 23 °C (Fig. 3A and B).

Fig. 3A shows the linear free energy relationships for the ATPase activities of MDR1 and PGP1 in the absence (basal ATPase) and presence (activation phase) of activating concentrations of various transport substrates. Two distinct regression lines were found representing two independent transition states. The solid line illustrates the linear regression for all the activating drugs concentrations used and represents the rate-limiting transition state for drug activation of ATPase activity. This transition state is common to both P-glycoproteins in both lipid environments and arises with all the classes of transport drugs tested. Thus, the transport of all drugs across Pgp is rate-limited by the same “global rate-limiting conformational coupling step.” On the other hand, the data for ATPase activity in the absence of drugs (basal activity) led to a statistically significantly different regression line (Fig. 3A, dashed line). This dashed line represents the uncoupled transition state in the absence of transport substrates. Clearly, basal and drug-activated ATPase activities have different rate-limiting transition states. This makes it unlikely that the basal ATPase is a consequence of an endogenous transport substrate. It is more likely a mechanistic property of P-glycoproteins.

The data for activating and inhibiting concentrations of transport drugs are plotted in Fig. 3B. In contrast to Fig. 3A, there is only one statistically significant regression line. Thus, inhibitory concentrations of drug do not lead to a new rate-limiting step and are still rate-limited by the same global rate-limiting conformational coupling step. The simplest explanation for this observation, consistent with transport results (23), would be that high concentrations of drug stop turnover by inhibiting the release of drug from the low affinity release site.

Correction of Thermodynamic Data to Saturating ATP—To correct the data to saturating ATP, the apparent \( K_{\text{m(A)}} \) values for each experimental condition were determined as a function of temperature. Fig. 4A illustrates the determination of the apparent \( K_{\text{m(A)}} \) as a function of temperature for the basal ATPase of MDR1 in mixed lipid proteoliposomes. Fig. 4B illustrates some examples of the variation in \( K_{\text{m(A)}} \) as a function of temperature for MDR1 in mixed lipid proteoliposomes. It is important to note that the apparent \( K_{\text{m(A)}} \) values vary significantly as a function of temperature. Apparent \( K_{\text{m(A)}} \) values in the range of 0.15–5 mrix were seen. In general, the \( K_{\text{m(A)}} \) decreased as a function of increasing temperature for drug activation of ATPase activity, but increased for basal ATPase activity. Different drugs also exhibited different \( K_{\text{m(A)}} \) profiles as a function of temperature. Overall, Fig. 4B emphasizes the complex nature of the coupling of the ATP hydrolytic reaction to the drug transport reaction.

Experimentally determined turnover numbers were then corrected to \( V_{\text{max(A)}} \) (turnover at saturating ATP) employing the experimentally determined apparent \( K_{\text{m(A)}} \) values to calculate enzyme saturation with ATP under the particular experimental conditions employed. These values were then replotted as Arrhenius plots (example plots are shown in Fig. 2B), from which enthalpic, entropic, and free energy terms could be calculated for saturating ATP (Tables II and III).

Deconvolution of Effects of Drugs on Pgp ATPase Activity—The drug-dependent ATPase activities of Pgp were fit to two different kinetic models described under “Experimental Procedures.” In Model 1 (Equation 1), the basal ATPase activity is independent of the activity associated with drug activation. In Model 2 (Equation 2), Pgp partitions between two active enzyme forms, an uncoupled form and a coupled form that transports drugs. In the absence of drugs, the entire enzyme exists in the uncoupled form, leading to basal ATPase activity. How-

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5 Some authors (e.g. Ref. 61) have observed very low basal activities starting from delipidated preparations. It is well established that these preparations need to be reactivated by the addition of lipids and reducing agents (62). We have found that the addition of transport drugs accelerates this process. Thus, starting ATPase assays with a delipidated preparation results in an underestimate of the true potential basal activity.
ever, as transport drugs are added and bind to the enzyme, more and more enzyme is recruited to the transport–competent coupled form at the expense of the uncoupled form. Thus, at saturating drug concentrations, the enzyme exists only in the coupled form. Previously, we found that at saturating concentrations of SL-verapamil, transport was strictly coupled (23). Fig. 5 shows fits of the colchicine-dependent ATPase activities of MDR1 in mixed lipids given in Fig. 1. The fit to Model 1 (Fig. 5A, solid line) is superimposable on the fit to Model 2 (Fig. 5B, solid line; $r^2 = 0.9867$ for both models). Clearly, the two models cannot be distinguished on the basis of kinetic data alone. In Fig. 3A, it was established that the rate-limiting step was different for basal ATPase and drug-activated ATPase activities. Also keeping in mind that activation and inhibition had the same temperature profile (Fig. 3B), it is possible to distinguish between the two models thermodynamically. Comparison of the Arrhenius data for basal activity and 2 mM colchicine activation of MDR1 in mixed lipids (Fig. 2, A and B) shows that the colchicine data intersects and crosses the basal data at low temperatures. In other words, activation of Pgp ATPase by colchicine at low temperatures appears to inhibit the basal activity. Clearly, the basal activity is not constant, as required by Model 1 (Equation 1), but is reduced by the presence of colchicine, indicating that Model 2 (Equation 2) is the correct one. This point is further illustrated in Fig. 3A, where the results of a colchicine titration (gray circles) cross below the basal ATPase linear free energy relationship (dashed line). Thus, 2 mM colchicine can appear to activate, have no effect, or inhibit ATPase activity depending on the temperature at which the assay is performed (Fig. 2, A and B), while all colchicine is being transported. Model 2 (Equation 2) resolves the paradox that some good transport substrates such as Hoechst 33342 appear to only inhibit ATPase activity (Table I). The use of Equation 2 should help reduce the confusion surrounding the dependence of ATPase activity on drug concentration encountered throughout the Pgp field. It is important to note that no cooperativity terms are needed for activation and inhibition by a single drug species (Equation 2). The appropriateness of Model 2 was further verified by fitting the kinetic results of a matrix of ATP, verapamil, and colchicine titrations to the "general modifier mechanism" of Botts and Morales (56). In such a mechanism, a drug acts as a hyperbolic activator or inhibitor of total Pgp ATPase activity. ATP acts as a hyperbolic activator of drug transport and transport-dependent ATPase. Purely on empirical grounds, Orłowski and co-workers (37, 57) concluded that Pgp follows the general modifier mechanism without defining the underlying mechanism. However, it should be pointed out that Model 2 (Equation 2) is a further extension of the general modifier mechanism that takes into account the effects of inhibitory concentrations of drugs on ATPase activity. If the effects of $K_i$ are ignored, as is the common practice (37, 57), erroneous estimates of the apparent $K_{m(D)}$ can arise. These errors can give rise to the appearance of moderate cooperativity and, in the extreme case, can lead to a reversed dependence of the apparent $K_{m(D)}$ on temperature (data not shown).

Correction of Thermodynamic Data to Saturating Ligand Concentrations—Drug titrations of ATPase activity were performed as a function of temperature, and the data were fitted to Equation 2. Using MDR1 in mixed lipids for verapamil and colchicine, the variation in apparent $K_{m(D)}$ as a function of temperature was plotted in the form of van’t Hoff plots (Fig. 6A). Analogous van’t Hoff plots are shown for the partitioning of the drugs between olive oil and aqueous buffer (Fig. 6B). The overall association of verapamil with the "activation sites" of Pgp at 35°C is exothermic (Fig. 6A), with a $\Delta^o H$ value of $-50.5$ kJ/mol ($\Delta^o S$ and $\Delta^o G$ values of $-25.5$ and $-25.0$ kJ/mol, respectively). For partitioning of verapamil into olive oil, the usual endothermic relationship for a "hydrophobic effect"-driven process was observed (Fig. 6B), with a $\Delta^o H$ value of $+27.4$ kJ/mol ($\Delta^o S$ and $\Delta^o G$ values of $+47.8$ and $-20.4$ kJ/mol, respectively). Together, these results imply (58) that the net driving force for verapamil binding to the activation sites of Pgp is specific noncovalent interactions such as hydrogen bonds. In contrast, for colchicine, the net driving force for binding to Pgp activation sites is hydrophobic interactions ($\Delta^o H$, $\Delta^o S$, and $\Delta^o G$ values of $+25.7$, $+44.0$ and, $-18.7$ kJ/mol, respectively) (Fig. 6A). For SL-verapamil (data not shown), specific noncovalent bond interactions were slightly more dominant over hydrophobic interactions ($\Delta^o H$, $\Delta^o S$, and $\Delta^o G$ values of $-18.6$, $+9.3$, and $-27.9$ kJ/mol, respectively). Thus, Pgp makes different types of binding interactions with different drugs, implying that different residues are involved in these interactions.

Apparent $K_{m(D)}$ values as a function of temperature were then obtained from the regression fits of the van’t Hoff plots (e.g. Fig. 6A). Turnover numbers at saturating ATP ($V_{\text{max,ATP}}$) were corrected to the intrinsic $k_{\text{cat}}$ at saturating ligand concen-
Steady-state Thermodynamics of P-glycoprotein

Corrected transition state thermodynamic parameters for steady-state ATP hydrolysis by human Pgp

Conditions were as follows: 35 °C, pH 7.5, and saturating ATP. Human Pgp (MDR1) was reconstituted into mixed lipid proteoliposomes. Values were calculated from the data sets shown in Table I. Values in parentheses are differences between parameters in the presence and absence of drugs. SL-verapamil, spin-labeled verapamil.

| Transport drug at given conc | ΔH[^b] | TΔS[^b] | ΔG[^b] | Stimulation (V[^b] / V[^b]baseline[^b]) | Drug specificity[^b] |
|-----------------------------|--------|---------|-------|--------------------------------------|---------------------|
| None (basal activity)       | 104.8  | 30.9    | 73.9  | -fold                                | 1 st[^b] s^-1       |
| 50 μM SL-verapamil          | 97.5 (7.3) | 27.5 (3.4) | 70.0 (3.9) | -fold                                | 1.03 x 10[^b]       |
| 130 μM verapamil            | 91.9 (12.9) | 21.3 (9.6) | 70.6 (3.3) | -fold                                | 0.87 x 10[^b]       |
| 130 μM valinomycin          | 108.3 (3.5) | 30.6 (5.7) | 71.7 (2.2) | -fold                                | 2.3 x 10[^b]        |
| 2 mM colchicine             | 112.8 (9.0) | 38.6 (7.7) | 74.2 (0.3) | -fold                                | 0.0028 x 10[^b]     |
| Saturating SL-verapamil     | 102.0 (2.8) | 32.8 (1.9) | 69.2 (4.7) | 6.2                                  | 0.63 x 10[^b]       |
| Saturating verapamil        | 104.1 (6.7) | 34.4 (3.5) | 69.7 (4.2) | 5.2                                  | 0.17 x 10[^b]       |
| Saturating valinomycin      | 108.3 (3.5) | 36.8 (5.7) | 71.7 (2.2) | 6.2                                  | 0.63 x 10[^b]       |
| Saturating colchicine       | 121.8 (17.0) | 48.3 (17.4) | 73.5 (0.4) | 1.2                                  | 0.0028 x 10[^b]     |

[^a] Intrinsic V[^a]max values for saturating substrates (V[^a]drug, V[^a]max for drug-dependent ATPase activity; V[^a]baseline, V[^a]max for basal ATPase activity).

[^b] Drug specificity constant as calculated by V[^b]drug/V[Kmolate].

Corrected transition state thermodynamic parameters for steady-state ATP hydrolysis by Chinese hamster Pgp

Conditions were as follows: 35 °C, pH 7.5, and saturating ATP. Chinese hamster Pgp (PGP1) was reconstituted into E. coli lipid proteoliposomes. Values were calculated from the data sets shown in Table I. Values in parentheses are differences between parameters in the presence and absence of drugs.

Transport Drugs Make Different Interactions with the Conformational Coupling Transition State—At saturating ligand concentrations, different drugs have different Arrhenius plots (Fig. 2C). Thus, the intrinsic k[^c]cat values for coupled drug transport are different for each transported drug even though they share the same rate-limiting coupling transition state (Fig. 3A). In other words, different drugs cause different interactions with the rate-limiting transition state. This situation is clearly different from that observed for the related ATP-binding cassette transporter TAP1/TAP2, which has a constant intrinsic k[^c]cat independent of the peptide species transported (59).

The results in Tables II and III show that there is an inverse relationship between the rate of drug transport (as indicated by ΔG[^c]) and activation energy (as indicated by ΔH[^c]) at saturating concentrations of ligands. As might have been predicted, drugs transported faster have higher intrinsic -fold stimulation of ATPase activity (V[^b]drug/V[^b]baseline) (Tables II and III). Thus, the intrinsic-fold stimulation of ATPase activity can be used as an indication of the tightness of interaction of the drug with the rate-limiting transition state, as postulated by Krujpa (60). In contrast, the drug specificity constant has no strong correlation with turnover rate (Tables II and III).

A convenient way to illustrate ligand or substituent effects on a transition state is to use an isokinetic plot, in which these changes are readily apparent (27). Fig. 8 shows an isokinetic plot for the intrinsic activation of ATPase activity in MDR1 and PGP1 by different transport substrates. The solid line is a representation of the compensation effect of substituents on the coupling transition state. According to the Eyring rate theory, points in the lower left corner have fewer bond interactions formed and broken during formation of the transition state compared with points in the upper right corner, which require more bond rearrangements. Rapidly transported drugs such as SL-verapamil are tightly bound to the transition state and require the protein to undergo fewer bond rearrangements than in the case of slowly transported substrates such as colchicine (Fig. 8). Solubilizing Pgp in the detergent 1-O-n-dodecyl
Fig. 5. Deconvolution of effects of colchicine on Pgp ATPase activity. The colchicine-dependent ATPase activities of MDR1 in mixed lipids given in Fig. 1 were fit to two alternative kinetic models, and the component kinetic parameters were plotted. A, data were fit to a non-partitioning kinetic steady-state model (Model 1, Equation 1; see “Experimental Procedures”) in which basal and drug-stimulated activities are independent entities. Components of the model include basal ATPase activity (no colchicine), colchicine-activated ATPase activity (at low activating concentrations), and colchicine-inhibited ATPase activity (at high inhibitory concentrations). B, data were fit to a partitioning kinetic steady-state model (Model 2, Equation 2) in which basal and drug-stimulated activities are dependent, and Pgp partitions between two forms: an uncoupled form and a coupled form in the presence of activating drug. The solid lines show the kinetic model fit to the data. The dotted lines show the inhibition of basal ATPase activity by high colchicine concentrations. The dashed and dotted lines show the activation of ATPase activities by activating concentrations of colchicine. The dashed lines show the inhibition of maximal ATPase activity by high colchicine concentrations. The fit parameters for A are as follows: $D = 222\%$, $K_{d,\text{ATP}} = 6.81 \text{ mm}$, $K_i = 18.5 \text{ mm}$, and $r^2 = 0.9867$. The fit parameters for B are given in the legend of Fig. 1.

β-D-maltopyranoside (Fig. 8) leads to a requirement for more bond rearrangements.6 We ascribe this effect to a loss of the structural constraints imposed by the organizing properties of the bilayer, leading to impaired carrier reorientation in detergent. This supports our postulate that the intrinsic rate-limiting step is a carrier reorientation step.

DISCUSSION

In this study, we employed kinetic and thermodynamic analyses that are new in the study of drug transport by Pgp. Using fully activated homogeneous preparations of Pgp reconstituted into specific transport-competent proteoliposomes, we were able to derive the intrinsic thermodynamic parameters of the intrinsic rate-limiting step of drug transport. This approach proved particularly revealing with respect to understanding the mechanism of drug transport and allowed us to derive an experimentally consistent kinetic scheme (Fig. 9). This kinetic scheme allows for more rigorous investigations of Pgp transport function. The results obtained were not species-dependent, as they were obtained with both human (MDR1) and Chinese hamster (PGP1) P-glycoproteins. In addition, we also resolved the apparent conundrum that good transport substrates can appear to be inhibitors of turnover (see “Results” and Figs. 2 and 3A). The techniques developed here allowed us to investigate the effects of Pgp mutations on the energetics of coupling (62) and to demonstrate that cholesterol is not a Pgp transport substrate.

Drug Control of ATPase Activity—We have confirmed that there are three phases of ATPase activity controlled by the concentration of transport drug present (Fig. 1). In the absence of transport drugs, there is an intrinsic basal ATPase activity. This activity is clearly rate-limited by a different transition state from the coupling transition state associated with drug activation (Fig. 3A). During drug transport, using many diverse and representative transport drugs (see “Results”), the ATPase activity was rate-limited by the same global coupling transition state (Fig. 3A). This was also the same rate-limiting step for SL-verapamil transport measured by a strict transport assay (see “Results”) (23). Clearly, the basal activity was not associated with drug transport in the classical sense. Thus, it is highly improbable that the transport of an unidentified molecule leads to the ATPase activity observed, as postulated by others (10–12). Even if there was an unidentified transport event associated with basal ATPase, such as a nonspecific lipid-flipping activity (60), it would still be operating on a different kinetic cycle, as illustrated in Fig. 9. Thus, basal ATPase, which has been observed in native plasma membranes (9), homogeneous purified and reconstituted preparations (9, 21, 47), and detergent-solubilized preparations (64), is an intrinsic feature of the mechanism of Pgp function. In contrast to basal activity, the inhibition of turnover of both ATPase (Fig. 1) and transport (23) by high concentrations of drug was rate-limited by the same step as for drug activation of turnover (Fig. 3B). No new kinetic paths were generated. Thus, high concentrations of drugs simply inhibit turnover by stopping release of drug from the low affinity release sites (23).

Overall Rate-limiting Transition State—Every chemical reaction and enzymatic step has an associated transition state. Thus, the apparent rate-limiting step of a transport cycle can change depending on the experimental conditions employed such that ATP binding or ADP release could become rate-limiting overall. In contrast, here we investigated the intrinsic rate-limiting transition state of drug transport when Pgp was fully saturated with activating ligands after accounting for any real inhibitory effects of ligands (such as the Ki associated with high drug concentrations). Under these conditions, the enzyme is not rate-limited by any ligand binding step because it is fully saturated. Employing SL-verapamil transport studies of the coupled cycle, we found that the transport step, and not the drug binding step, is rate-limiting overall (23). By further investigating force/flux relationships, we also showed that the actual chemical step of ATP hydrolysis was directly coupled to the change in drug binding affinities. This was not surprising, as we have shown previously that this step has the largest thermodynamic energy drop of any step within the catalytic mechanism (7, 65). From inspection of apparent $K_O$ values (e.g., Table 1), as is the usual practice (46–48), it is difficult to discern any meaningful trends that shed light on the mechanism, as they are all condition-dependent. Only after recalcu-

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6 The intrinsic $K_{m,\text{act}}$ values with saturating ligand concentrations at 35 °C for MDR1 solubilized in 2 mM 1-O-n-dodecyl β-D-maltopyranoside were 2.1 and 1.7 s⁻¹ for basal and valinomycin-stimulated activities, respectively. The apparent inhibition was analogous to what was seen with colchicine at low temperatures and further illustrates the hyperbolic nature of drug activation or inhibition of ATPase activity.
lating these observations to intrinsic thermodynamic parameters of Pgp (Fig. 7 and Tables II and III) do mechanistic trends reveal themselves. Both basal and coupled ATPase activities had large intrinsic activation enthalpies compensated by similarly large activation entropies (Tables II and III). This makes it highly likely that the rate-limiting coupling transition state entails a large conformational rearrangement (see “Results”). Similarly large activation energies were observed in the opening of the cystic fibrosis transmembrane conductance regulator (CFTR) ion channel upon ATP binding (66). A very important finding of the present study is that transport of all the drugs was rate-limited by the same global rate-limiting conforma-
The coupled cycle is the tightly coupled alternating catalytic cycle described previously (7, 23). At full drug saturation of the loading site, all Pgp molecules enter the coupled cycle. For coupled activity, the transport drug binds first, followed by ATP to form the ternary complex. Binding of this complex facilitates the next catalytic cycle by Pgp, thereby allowing ATP to hydrolyze without any transport work. However, if there is insufficient drug, and two ATP molecules bind (i.e., the second ATP molecule binds before a drug molecule binds), Pgp partitions to the uncoupled cycle and hydrolyzes ATP without any transport work.

The global rate-limiting step is associated with single fixed molecular interactions with this rate-limiting transition state such that the intrinsic $k_{cat}$ of Pgp is different with each drug employed (Figs. 2 and 8 and Tables II and III). Additionally, the fact that each drug had a different intrinsic $k_{cat}$ value implies that the drugs were still bound at the time the rate-limiting transition state was formed in the forward direction as measured here (see also Ref. 67). Different molecular interactions were also apparent in the van’t Hoff plots of Fig. 6. It was thus found that drugs that tightly bound the transition state required fewer conformational rearrangements by the protein to achieve the transition state (see “Results,” Figs. 8, and Tables II and III). Consequently, these drugs were transported faster with a higher degree of intrinsic coupling. Recently, we showed that the Pgp mutation G185V improves colchicine transport by enhancing its binding to the coupling transition state (62).

There is a broad consensus (67–70) that in coupled transport cycles of Pgp, the step of drug release precedes the step of ADP release (Fig. 9). As the coupling transition state still had drug bound to it (see above), it was clear that the ADP release step was not the intrinsic rate-limiting step of transport, as proposed by others (68). Also, the fact that the different drugs led to different intrinsic $k_{cat}$ values further reduces the probability that the global rate-limiting step is associated with single fixed steps such as Pi or ADP release. Although not the focus of this study, lipids and detergents had profound effects on the coupling. Recently, we showed that drugs that tightly bound the transition state required fewer conformational rearrangements by the protein to achieve the transition state (see “Results,” Figs. 8, and Tables II and III). Consequently, these drugs were transported faster with a higher degree of intrinsic coupling. Recently, we showed that the Pgp mutation G185V improves colchicine transport by enhancing its binding to the coupling transition state (62).
pling transition state (see “Results” and Fig. 8). Thus, the coupling transition state was directly sensing the lipid environment. Taken together, all the facts discussed above make it highly likely that the intrinsic overall rate-limiting-step is a concerted conformational change (Fig. 9, red star in the coupled activity cycle). Recently, Qu et al. (67) confirmed our postulate of the existence of such a global conformational coupling transition state (7). We originally postulated that drug-dependent ATP hydrolysis by Pgp generated a high energy intermediate (global conformational coupling transition state) that, upon relaxation, would drive the drug across the membrane. An analogous global conformational coupling transition state has been demonstrated in the E. coli maltose transporter (ATP-binding cassette transport protein MalGPK3-MBP complex) (71).

**Kinetic Pathway**—We previously proposed that Pgp employs an alternating catalytic site mechanism with symmetrical ATP hydrolysis at two kinetically indistinguishable, but physically non-identical nucleotide-binding sites (65). This idea has survived the test of time and is broadly accepted as the mechanism by which Pgp operates (72). However, this mechanism is not universal for all ATP-binding cassette transport proteins. Both cystic fibrosis transmembrane conductance regulator and multidrug resistance proteins (phylogenetically related families) appear to utilize a nonsymmetrical ATP hydrolytic mechanism in which the two nucleotide sites are kinetically nonequivalent (73–75). In the absence of transport substrates, Pgp can bind two molecules of ATP (70), and drug and ATP can bind in random order. Thus, Pgp can enter the uncoupled activity pathway illustrated in Fig. 9 (shaded). In general, we found that the $K_{m}(A)$ values for the coupled pathway were lower than the $K_{m}(A)$ values for the uncoupled pathway, but there were some exceptions such as colchicine (Fig. 4B). This is one way in which Pgp reduces the physiological degree of uncoupled ATPase activity.

There is more than adequate energy in the hydrolysis of one ATP molecule to drive transport. In cells, the free energy of ATP hydrolysis values are in the range of $\sim 42$ to $\sim 54$ kJ/mol (76). The free energy of rehydrating a drug bound to the high affinity site of Pgp can be calculated. This is the ultimate energy required for drug transport. We have calculated these free energy values (data not shown) for a series of transported drugs and found them to be significantly lower than $39.8$ kJ/mol. The value is for cyclosporin A, a high affinity transport substrate.

As discussed by Urbatsch et al. (72), the alternating catalytic site mechanism retains a “conformational memory” of the previous reaction cycle in the form of a previously bound ATP, which allows the two nucleotide sites to be functionally equivalent. The kinetic scheme as illustrated in Fig. 9 obviates the need for an independent drug site reset step coupled to the hydrolysis of one ATP molecule as proposed by Sauna and Ambudkar (14). In our scheme (Fig. 9), the drug-binding sites reset automatically after the dissociation of ADP by forming a mobile carrier form of the protein.

**Physiological Control of ATPase Activity**—The question arises as to why Pgp employs such an unusual transport mechanism (Fig. 9). A large component of the drug selectivity of this protein arises from partitioning of hydrophobic transport drugs into the inner leaflet, followed by protein binding (hydrophobic vacuum cleaner model) (77). The partitioning between these two phases (membrane inner leaflet and high affinity drug-binding sites) is nearly isoequemonic (78). Thus, the interaction of the drug with Pgp is weak relative to the membrane. This may be a strict requirement for low drug selectivity by Pgp to maximize the chemical entities that can be transported. Given that Pgp functions physiologically as a toxic xenobiotic exporter, and given the high toxicity of its substrates, Pgp has evolved to maximize successful drug transport. We postulate that the central ATP$^E_2$ species of Fig. 9 (located in both the coupled and uncoupled cycles) is a mobile carrier form of the protein. In this species, the high and low affinity drug-binding sites are in equilibrium, and this species is the only species that is competent to bind drugs for transport. Thus, its concentration must be maximized. If Pgp binds two ATP molecules in the presence of high ATP and low drug concentrations, it becomes kinetically incompetent for transport. Under such conditions, the dissociation of ATP is slow and unlikely. Given that the basal ATPase activity of Pgp (see “Results”) can be inhibited by high drug concentrations, it is likely that the drug sites are in a low affinity form in the uncoupled cycle (Fig. 9, shaded). Thus, Pgp has evolved the uncoupled ATPase cycle (Fig. 9, shaded) to return quickly to the transport-competent form ATP$^E_2$ and to maximize its concentration. In other words, Pgp has evolved a parallel process that is always faster than a serial process that is rate-limited by the slowest step. Clearly, Pgp pays the price of this cellular protection vigilance with lowered overall coupling efficiency. Energetically speaking, Pgp works as a drug rehybridation machine. It is thus easy to envisage a mechanism of drug transport entailing the rotation of transmembrane helices such that water is allowed to enter and interact with the protein drug-binding site. At the same time, favorable specific bond interactions (see “Results”) that the protein has with the transported drug are abolished by these same helix-rotating events. As a consequence of these conformational changes, the affinity of drug for Pgp is reduced, and the drug is located in an aqueous environment. Experiments are currently under way to test these hypotheses.

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