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The Occluded Nucleotide Conformation of P-Glycoprotein

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We review recent work on E552A/E1197A P-glycoprotein. This ATPase-defective mutant occludes MgATP tightly with maximal 1/1 stoichiometry in drug-sensitive fashion. The occluded nucleotide conformation appears to represent a transient, asymmetric, catalytic intermediate. We present a model for catalysis incorporating nucleotide binding domain (NBD) dimerization and the occluded nucleotide conformation, and we speculate as to how catalysis seen in P-glycoprotein might be harmonized with symmetrical dimer structures of isolated NBDs.

KEY WORDS: P-glycoprotein; ATPase catalytic mechanism; catalytic carboxylates; MgATP binding; occluded nucleotide conformation.

INTRODUCTION

The first model for P-glycoprotein (Pgp) catalytic mechanism postulated that the two nucleotide-binding domains (N-terminal NBD1 and C-terminal NBD2) hydrolyze ATP in an alternating sequence, with drug transport linked to relaxation of a high-energy NBD conformation generated by ATP hydrolysis (Senior *et al*., 1995). Since that time a great deal has been learned about Pgp mechanism in many laboratories, however, high-resolution structural analysis remains elusive, and so progress toward definition and refinement of Pgp mechanism has been delayed. A theme that has emerged recently from X-ray crystallography of ABC transporters is "dimerization" of the two NBDs. Isolated NBD subunits or domains from diverse transporters (all of which were inactive in ATP hydrolysis either via mutation or lack of Mg^{2+}-cofactor) have been visualized as symmetric dimers with two molecules of ATP bound at the dimer interface (Chen *et al*., 2003; Hopfner *et al*., 2000; Smith *et al*., 2002; Zaitseva *et al*., 2005a). Biochemical studies of isolated NBDs likewise have identified dimer species with 2 mol of nucleotide bound per dimer (Horn *et al*., 2003; Janas *et al*., 2003; Moody *et al*., 2002; Verdon *et al*., 2003; Zaitseva *et al*., 2005b). There appears to be consensus that the NBD dimers represent a catalytic intermediate. Biochemical, mutagenesis, and low resolution structural studies have supported the idea of NBD dimerization in Pgp (Lee *et al*., 2002; Loo *et al*., 2002, Rosenberg *et al*., 2005; Urbatsch *et al*., 2000a, 2001). In the X-ray structure of nucleotide-free, intact BtuCD (with transmembrane domains, TMDs, present), the NBDs were closely apposed in symmetrical manner (Locher *et al*., 2002). However, a structure of MsbA in the ADP-Vi trapped state was clearly asymmetric (Reyes and Chang, 2005), reflecting extensive earlier biochemical literature on Pgp showing that ADP-trapping by Vi or BeFx occurs at only one of the two potential catalytic sites (Sankaran *et al*., 1997; Urbatsch *et al*., 1995). We recently found that Pgp containing Glu to Ala mutations at both "catalytic carboxylate" residues (E552A in NBD1 and E1197A in NBD2) occludes ATP at just one site. Here we review properties of this mutant and derive a hypothesis that attempts to harmonize the data discussed above into a unified catalytic mechanism.

RESULTS AND DISCUSSION

The data on E552A/E1197A mutant Pgp have been published in Tombline *et al*. (2004a, 2005). Wild-type

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Abbreviations: Pgp, P-glycoprotein; NBD, nucleotide binding domain; TMD, transmembrane domain; Vi, orthovanadate; BeFx, beryllium fluoride.

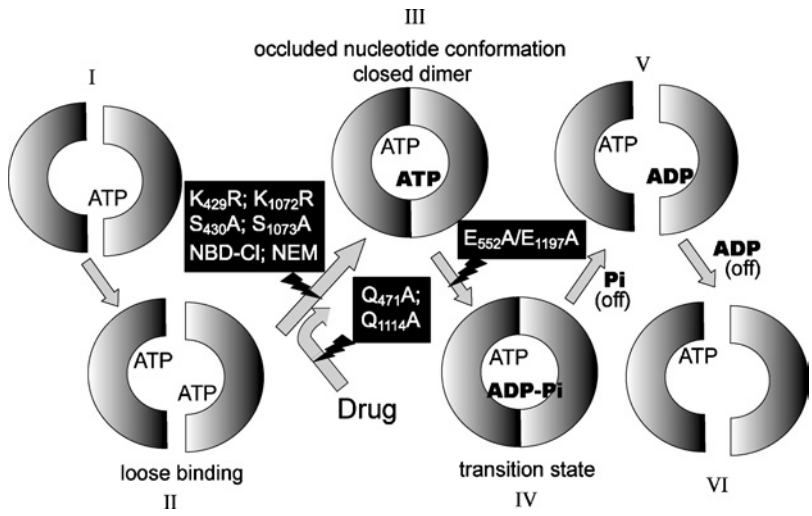

Fig. 1. Proposed model for the catalytic pathway of Pgp, incorporating the occluded nucleotide conformation. The catalytic pathway is shown as a series of intermediate *states I–VI* as discussed in the text. The occluded nucleotide conformation occurs at *state III* with the ATP that is bound tightly and (we hypothesize) committed to hydrolysis (in bold). Steps that are suggested to be blocked by mutations or chemical modification are indicated, as also is acceleration by drug. Lys-429, Lys-1072, Ser-430 and Ser-1073 are Walker A residues involved in catalysis; NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3,-diazole) and NEM (*N*-ethylmaleimide) are potent covalent inhibitors of ATPase activity; Gln-471 and Gln-1114 are "Q-loop" residues thought to be involved in TMD-NBD interdomain communication.

Pgp binds MgATP weakly ($K_d \sim 0.4$ mM; $K_m \sim 0.3$ mM, Qu *et al*., 2003; Tombline *et al*., 2004b) and does not retain any nucleotide after centrifuge column elution. In contrast, pure E552A/E1197A Pgp retains maximally 1 mol/mol nucleotide upon centrifuge column elution after preincubation with MgATP, indicating $k_{off} \leq 0.006\ s^{-1}$. The apparent K_d(MgATP) is 9 μM and the Hill coefficient is 1.6. Tightly bound nucleotide is predominantly in the form of ATP, with about 20% ADP, consistent with the very low ATPase activity of the mutant Pgp ($0.012\ s^{-1}$). Tight binding of nucleotide was Mg^{2+}-dependent. Verapamil stimulates nucleotide occlusion by increasing the apparent association rate, however it neither affects final stoichiometry, nor dissociation rate of MgATP. Other Pgp drugs and modulators also promoted nucleotide occlusion. Prereaction with *N*-ethylmaleimide or 7-chloro-4-nitrobenzo-2-oxa-1,3,-diazole, two potent inhibitors of ATPase activity, strongly disfavored nucleotide occlusion. It had been found earlier that single mutations of important catalytic Ser and Lys residues in the Walker A sequences of either NBD1 or NBD2 were sufficient to block ATP hydrolysis and ADP-Vi trapping (Urbatsch *et al*., 1998, 2000a); combination of these mutations with E552A/E1197A abrogated tight binding of MgATP. Also, earlier work had indicated that mutations of the "Q-loop" Gln residues impaired interdomain communication between TMDs and NBDs (Urbatsch *et al*., 2000b), as measured by drug-stimulation of ATPase. When either of these mutations (Q471A or Q1114A) was combined with E552A/E1197A, tight binding of MgATP was strongly impaired.

Together this data suggested that both NBDs are involved in the tight binding of MgATP, even though the maximal stoichiometry of binding was 1 mol/mol. While not proven by structural analysis, it seems likely that the "occluded nucleotide conformation" is the result of NBD dimerization, revealed in E552A/E1197A mutant Pgp because the catalytic pathway is attenuated by removal of the catalytic Glu residues.

In Fig. 1 we propose a model for the catalytic pathway of Pgp that incorporates NBD dimerization and the occluded nucleotide conformation. Initial loose binding of MgATP at both NBDs and of drug at the TMD drug-binding site triggers formation of a closed dimeric NBD conformation (*states I* $\rightarrow$ *II* $\rightarrow$ *III*). *State III* is the form seen in E552A/E1197A Pgp, in which one ATP is bound very tightly (in bold). We hypothesize that this ATP is committed to hydrolysis. In wild-type Pgp, catalysis would proceed rapidly with the tightly bound ATP entering the transition state and forming ADP and Pi (*states III* $\rightarrow$ *IV*). Then Pi and ADP are released as the dimer opens (*states V*, *VI*) and binding of new ATP from the

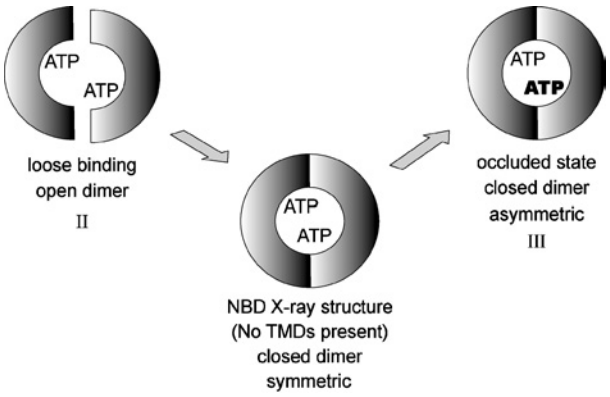

Fig. 2. Where do X-ray structures showing symmetrical dimers of isolated NBDs fit in? We propose that the symmetrical dimers of isolated NBDs represent a transient intermediate that occurs after *state II* of Fig. 1, and which in intact transporter would rapidly progress to *state III*.

medium would occur. Steps at which mutations, chemical modifications, and drugs appear to impact the pathway are shown in Fig. 1.

If the nonhydrolyzed ATP is retained after release of product ADP (as suggested in the model, *state VI*), such that new ATP has time to bind, then hydrolysis in alternating sites (NBDs) could be operative. A recent molecular dynamics simulation of BtuCD conformational changes favored such a scenario (Oloo and Tieleman, 2004).

This same molecular dynamics simulation supported the idea that after initial binding of MgATP in intact BtuCD, the NBD dimer interface moves toward an asymmetric state in which one of the two nucleotides progressively becomes more tightly bound. It is also noteworthy that, as with Pgp, in intact BmrA mutated at the catalytic carboxylate, a nucleotide binding stoichiometry of 1 mol/mol dimer was seen (Orelle *et al*., 2003). Thus it appears that presence of the TMDs promotes formation of an asymmetric state. Where then do symmetric dimers of isolated NBDs fit in? Figure 2 shows how the data might be harmonized. *States II* and *III* are the same as in Fig. 1, but we include an intermediate that conforms to the dimers seen in X-ray crystallography of isolated NBDs, i.e., it binds MgATP symmetrically. We suggest that this intermediate occurs, fleetingly, in intact transporters such as Pgp, and that presence of TMDs and bound drug speeds progression to *state III* and subsequently to the transition state of catalysis.

While ATPase activity is seen in preparations of isolated NBDs, it appears to differ in important ways from that found in intact transporters. Obviously, it is not coupled to transport substrate binding or to the conformational changes involved in transport. It usually has slow turnover, too slow in fact to account for physiological rates of transport. For example, in the well-characterized maltose transport system, coupled intact transporter ATPase ($\sim$11 s^{-1}) turns over 100 times faster than that of isolated MalK NBD dimer ($\sim$0.1 s^{-1}) (Chen *et al*., 2001; Sharma and Davidson, 2000). Further, the ATPase activity of isolated NBDs is often Vi-insensitive, in contrast to that of the corresponding intact transporter (e.g., Sharma and Davidson, 2000). This suggests that isolated NBDs do not utilize the same catalytic pathway and transition state structure as do intact transporters. Speculatively, the closed symmetric isolated NBD dimer shown as an intermediate following state *II* in Fig. 2 cannot proceed rapidly to *state III*, as it would in intact transporter, but relaxes instead via whatever catalytic pathway it can find. Future structural work using transition state analogs in intact transporters and isolated NBDs may throw light on this question.

The emergence of the concept of NBD dimerization in catalysis in ABC transporters could lead to discovery of a new family of small molecule or peptide inhibitors that bind tightly at the dimer interface.

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