Properties of P-glycoprotein with mutations in the "catalytic carboxylate"

glutamate residues

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Footnote 1. Pgp, P-glycoprotein; NBD, nucleotide binding domain; Vi, orthovanadate; DTT, dithiothreitol.

Footnote 2. This conclusion was affirmed by unpublished work in which we mutated Glu556 and Glu1201 to Cys in otherwise Cys-less human MDR1 Pgp (residues 556 and 1201 in human MDR1 are equivalent to 552 and 1197 in mouse mdr3). The Cys mutations resulted in very low rates of ATPase. Covalent reaction of the Cys with iodoacetic acid did not regenerate activity.

Footnote 3. Given the very low turnover rate evident for mutant E552A/S528A in Fig. 6B the fact that ADP was bound to Pgp might seem surprising. However, an ATP hydrolysis turnover rate of e.g. 0.001 s\(^{-1}\) would cause only 0.05 nmol of ATP to be hydrolysed in 240 min in Fig. 6B, which is at the detection limit, and would produce 1.2 turnovers per Pgp during the 20 min incubation of Fig. 7.
ABSTRACT

It is known from earlier work that two conserved Glu residues, designated "catalytic carboxylates", are critical for function in P-glycoprotein (Pgp). Here the role of these residues (Glu552 and Glu1197 in mouse mdr3 Pgp) was studied further. Mutations E552Q or E1197Q reduced Pgp-ATPase to low but still measurable rates. Two explanations previously-offered for effects of these mutations, namely that ADP release is slowed, or that a second (drug-site-resetting) round of ATP hydrolysis is blocked, were evaluated and appeared unsatisfactory. Thus the study was extended to include E552A, D, K, and E1197A, D, and K mutants. All reduced ATPase to similar low but measurable rates. Vi-trapping experiments showed that mutation to Gln, Ala, Asp, or Lys, altered characteristics of the transition state, but did not eliminate its formation, in contrast e.g. with mutation of the analogous catalytic Glu in F1-ATPase. Retention of ATP as well as ADP was seen in Ala, Asp and Lys mutants. Mutation E552A in NBD1 was combined with mutation S528A or S1173A, in the LSGGQ sequence of NBD1 or NBD2, respectively. Synergistic effects were seen. E552A/S1173A had extremely low turnover rate for ATPase, while E552A/S528A showed zero or close to zero ATPase. Both showed Vi-independent retention of ATP and ADP. We propose that mutations of the catalytic Glu residues interfere with formation and characteristics of a closed conformation, involving an interdigitated NBD dimer interface, which normally occurs immediately following ATP binding and progresses to the transition state.
INTRODUCTION

Expression of P-glycoprotein (Pgp\(^1\)) in plasma membranes of cells causes resistance to multiple, structurally-diverse drugs in cancer and AIDS patients (reviewed in 1,2). Pgp is a member of the ABC transporter family. It confers drug-resistance by coupling ATP binding and hydrolysis at two cytoplasmic nucleotide binding domains (NBDs) to drug export via two transmembrane domains. The first mechanistic model for drug transport (3) proposed that ATP is hydrolysed alternately at the two NBDs, with collapse of the chemical transition state and release of product Pi providing driving force for drug export via coupled long-range conformational changes between the NBDs and the transmembrane domains. This model has gained acceptance as a general framework, with recently suggested modifications and refinements (4,5). Defining the mechanism of Pgp in detail is an important goal, because it relates to conceiving strategies to disable or circumvent the action of Pgp in patients and to understanding the mechanism of ABC transporters generally.

Biochemical and mutagenesis studies showed that the NBDs of Pgp act cooperatively (3) and suggested that they alternate between "open" and "closed" conformations (6,7). We have proposed that ATP initially binds to each of the NBDs while Pgp is in the open conformation, then the NBDs approach each other to form an integrated structure, the closed conformation, in which catalytic side-chains required to stabilize the ATP hydrolysis transition state complex are made available from both NBDs. After hydrolysis of ATP coupled to drug movement from inner membrane leaflet to the outer side of the membrane, the NBDs dissociate to re-form the open
conformation then release ADP product (6,7). Data showing binding of ATP at two sites in Pgp with relatively weak affinity (8) provided evidence for an open conformation, while evidence for a closed conformation consisting of interdigitated NBDs has come from crosslinking studies (9-11), and from a recent study in which combined mutagenesis of the Pgp NBDs produced an occluded conformation which binds ATP and ADP very tightly (see below). In other ABC transporters there is extensive evidence for dimerization of the NBDs, producing a conformation which binds nucleotide at the interface between two NBDs. Examples include X-ray structural data (12-15), photocleavage studies (16), and gel filtration studies of NBD subunit dimers (17-19). Mutagenesis of specific conserved Glu residues termed "catalytic carboxylates" facilitated NBD dimerization in several of these studies, while at the same time reducing ATPase activity to very low rates.

In several, diverse, ATPase enzymes Glu residues have been implicated from their location in X-ray structure models of the catalytic sites to play a critical role in ATP hydrolysis by immobilizing, polarizing, and stereochemically-orienting a water molecule in position for in-line nucleophilic attack at the γ-P of ATP (20). Prominent early examples were recA protein (21) and F₁-ATPase (22). Biochemical evidence has supported this concept. For example, in F₁-ATPase the relevant Glu has been shown to be directly involved in bond formation in the transition state complex (23-25), and modulation of the carboxylate side-chain location by substitution with Asp or iodoacetic acid-reacted Cys gravely impaired catalysis (26). Beginning with the X-ray structure of HisP NBD subunit (27), and later in several other X-ray structures, it was apparent that a conserved Glu residue immediately adjacent to and downstream of the conserved
"Walker B" Asp residue might provide the same catalytic carboxylate function in ABC transporter NBDs.

A mutagenesis screen of carboxylate residues by Urbatsch et al. (28) underscored the importance of the highly-conserved, putative catalytic carboxylate residues Glu552 and Glu1197 in mouse mdr3 Pgp. Mutagenesis of either residue to Gln yielded Pgp with strongly-impaired drug transport and ATPase activities, although mutant proteins did show vanadate (Vi)-induced trapping of 8-azido-ADP upon incubation with 8-azido-ATP and Vi, suggesting that at least one turnover of 8-azido-ATP hydrolysis as far as the Pi release step was occurring. Urbatsch et al. proposed that in the mutants, release of product ADP was unusually slow (28). Later studies from the same laboratory indicated that mutants E552Q and E1197Q induce asymmetric conformations of the two NBDs (29,30). Sauna et al. (31) studied the equivalent conserved glutamates in human mdr1 Pgp, introducing Gln and Ala mutations. Consistent with the earlier data, both drug transport and ATPase activity were strongly-impaired in mutant proteins, and Vi-induced trapping of 8-azido-ADP was seen. However, these workers differed from Urbatsch et al. as to the impact of the mutations, concluding that a second round of ATP hydrolysis, required to "reset" the drug-binding sites after an initial outward-transport-generating ATPase turnover, was blocked. Sauna et al. (31) also found that when Ala or Gln mutations were introduced simultaneously into both NBDs, the resultant double mutants showed Vi-independent occlusion of 8-azido-ADP upon incubation with 8-azido-ATP, along with loss of ATPase and drug transport.
In a recent study we further characterized nucleotide binding and hydrolysis properties of pure mouse mdr3 Pgp in which Glu residues at both positions 552 and 1197 were simultaneously substituted with Ala, Gln, Asp, or Lys (32). Using a sensitive radioactive assay, we found that the mutants displayed real but very low ATPase activity, and that inability to form the normal transition state, rather than slow ADP release, was the primary defect. Both E552Q/E1197Q and E552A/E1197A mutants were able to bind MgATP and MgADP with high affinity, suggesting that they were trapped in the closed conformation of Pgp which, we propose, occurs immediately before the hydrolytic step (see above).

Therefore it was apparent that mutations of the catalytic carboxylate glutamate residues in Pgp have the potential to clarify several aspects of mechanism. Here we present detailed studies of effects of mutations Glu to Gln, Ala, Asp or Lys, present singly in either NBD, using pure mouse mdr3 protein and the naturally-occurring ligands MgATP and MgADP. First we evaluated two previous suggestions, namely that ADP release (28), or a second round of ATPase turnover (31), is defective in Gln mutants. Second, we extended the studies using Ala, Asp and Lys mutants. Third, we combined the mutations S528A and S1173A, involving mutations of the conserved Ser residue in the "LSGGQ" ABC signature sequence of NBD1 or NBD2, with the catalytic carboxylate E552A mutation of NBD1, to assess the effects of mutations "in cis" or "in trans" at the putative NBD dimer interface.
EXPERIMENTAL PROCEDURES

Materials. [8-14C]ADP and [α-32P]ATP were purchased from Perkin Elmer Life Sciences. *Escherichia coli* lipids were purchased from Avanti Polar Lipids.

Construction of mutant mouse mdr3 Pgp; Expression of mutant Pgp in *Pichia pastoris*; Purification and quantitation of Pgp. *Pichia pastoris* strains expressing E552Q and E1197Q mutant mouse mdr3 Pgp were generous gifts of Professor Philippe Gros and Dr. Isabel Carrier (McGill University). Other mutations at positions Glu552 and Glu1197 were generated by PCR mutagenesis as described previously (32). Mutants E552A/S528A and E552A/S1173A were generated by transferring a BglII-Smal fragment containing the S528A mutation or an Spe1-SnaB1 fragment containing S1173A (33) into pHILD-mdr3.6-His6 containing E552A (32). All mutant proteins contained a C-terminal 6-His tag, and were expressed in *P. pastoris* and purified as described previously (32,33). Protein concentration was determined by reference to a standard calibrated by amino acid analysis as described previously (33).

Activation of Pgp by DTT and lipids; Assay of ATPase activity; Centrifuge column elution of Pgp. This was as previously described (32,33). Briefly Pgp was activated by preincubation with 8 mM DTT and *E. coli* lipids at ratio of 50/1 (for ATPase measurement) or 2/1 (for nucleotide trapping experiments). Release of [32P] Pi from [γ-32P]ATP was determined using the charcoal (Norit) adsorption assay using 0.5 - 1 µg activated Pgp. When present, verapamil (150 µM) was added directly to the ATPase assay buffer. $K_m$(MgATP) and $K_i$(MgADP) were determined as in (32,33).
Trapping of nucleotide by vanadate (Vi). Trapping of nucleotide by Pgp after preincubation with [α-32P]ATP or [8-14C]ADP and Vi utilized centrifuge column elution as described previously (32,33). Briefly the trapping procedure entailed preincubation of Pgp (5-10 µg) with 200 µM [α-32P]ATP or 100 µM [8-14C]ADP, 200 µM Vi, and 2 mM MgSO₄, with or without 150 µM verapamil, at 37°C for 20 min (with ATP) or 120 min (with ADP), followed by centrifuge column elution to separate bound from free ligands, and counting of eluates to determine bound nucleotide. Identification of the trapped nucleotide (ATP vs ADP) was by TLC analysis (32). Rate of onset of trapping was carried out at room temperature, to slow the reaction sufficiently to allow accurate determination. Rate of release of trapped nucleotide was measured by allowing centrifuge column eluates to incubate for varied times at 37°C, then passing through a second centrifuge column to determine remaining bound nucleotide. Where sequential rounds of Vi trapping were carried out the procedure was modified as follows. In the first round of Vi-trapping, 90 µg activated Pgp was incubated in 40 mM Tris-HCl pH 7.4, 0.1 mM EGTA, 2.5% glycerol, 8 mM DTT, 4 mM MgSO₄, with 150 µM verapamil, 200 µM [α-32P]ATP and 200 µM Vi at 37°C for 20 min in a total volume of 200 µl. Samples were placed on ice for 2 min, then 2 x 100µl aliquots were passed through centrifuge columns at 4°C that were pre-equilibrated with 40 mM Tris-HCl pH 7.4, 0.1 mM EGTA, 10% glycerol, 10 mM DTT. The eluates were combined, quickly counted by the Cerenkov method, and a 10 µl sample was removed for protein quantitation. MgATP was added to a concentration of 1 mM. The eluates were then incubated at 37°C for 180 min (wild-type and E552Q) or 90 min (E1197Q) to allow release of trapped nucleotide. Next, samples were placed on ice for 2 min followed by passage through a
centrifuge column equilibrated as above. After quickly counting by the Cerenkov method, 5 µl was removed for protein quantitation. For the second round of Vi-trapping, remaining sample was incubated under conditions identical to the first round of trapping, in 100 µl total volume at 37°C for 20 min. Samples were then placed on ice for 2 min followed by passage through centrifuge columns at 4°C. Eluates were counted by the Cerenkov method and protein was quantitated. It was noted that Pgp (wild-type or mutant) lost activity if DTT or ATP was omitted at any of the centrifuge column steps.
RESULTS

General.  As remarked in INTRODUCTION, there is agreement that the conserved Glu residues (Glu552 and Glu1197 in mouse mdr3 Pgp) that lie immediately adjacent and C-terminal to the Walker B Asp residues in the NBDs are catalytically-important in P-glycoprotein. However, previous authors differed as to the mechanism by which mutations to Gln impaired function (28,31). Here we further studied the roles of these Glu residues. First we re-evaluated the earlier conclusions; second we made mutations to Ala, Asp and Lys and we compare data obtained with these mutants; third, we combined the E552A mutation in the N-terminal NBD with a mutation Ser to Ala in the LSGGQ ABC signature sequence of either the N-terminal or the C-terminal NBD. Pure mouse mdr3 Pgp obtained from Pichia pastoris grown in fermentor culture was used throughout. Yields of all mutant proteins were the same as for wild-type Pgp. All purified proteins showed a single band on SDS-gels, as for example in (33).

1. Effects of E552Q and E1197Q mutations.

ATPase activity.  Pgp containing E552Q and E1197Q mutations, in the N- and C-terminal NBDs respectively, was purified and activated with lipids and DTT. ATPase activity was measured using [γ-32P]ATP in a sensitive charcoal-adsorption assay. Results are shown in Figure 1A,B (note differences in vertical axes). Calculated turnover rates are given in Table 1, with wild-type enzyme assayed under the same conditions shown for comparison. Duplicate purifications of each mutant enzyme gave
the same results. Contrary to previous results (28,31) we find that both E552Q and E1197Q mutants undergo multiple turnovers during the time-course of the assay (contrast these data with the zero or close to zero activity of mutant E552A/S528A shown in Figure 6B, also reproduced in duplicate Pgp purifications). Moreover there was apparent "asymmetry" in that the mutation in the C-terminal NBD (E1197Q) was more impairing than the one in the N-terminal domain (E552Q), in regard to turnover and drug stimulation. As with wild-type, both mutant enzymes showed activation and then inhibition when titrated with increasing concentration of verapamil (data not shown). Concentration of verapamil yielding maximal activity was \(~150 \mu M\) for all three enzymes. Vanadate (Vi) potently inhibited wild-type and both mutant enzymes. IC\(_{50}\) values were: wild-type, 3 \(\mu M\); E552Q, 0.1 \(\mu M\); E1197Q, 0.2 \(\mu M\) (data not shown). The enhanced apparent affinity for Vi in mutants is consistent with previous work (30). Km values were calculated from plots of ATPase activity vs. MgATP concentration (Figure 2) and are given in Table 1. Since for technical reasons the maximal ATP concentration used was 2 mM, which was insufficient to reach saturation in Fig 2A,B, these values are apparent only. However they are useful for comparisons, showing both mutants had lower Km than wild-type, with E1197Q producing a larger effect.

**Determination of K\(_a\)ADP.** Figure 2 shows ATPase activity in presence of varied concentration of MgADP, from which K\(_a\)(MgADP) was calculated (Table 1). It is seen that the E552Q and E1197Q mutants were not very different from wild-type, indicating that ADP binding affinity is not significantly altered by the mutations.
Release and formation of Vi-trapped ADP. Figure 3 shows release of $[\alpha^{32}\text{P}]\text{ADP}$ after prior trapping with Vi and $[\alpha^{32}\text{P}]\text{ATP}$. Rates and half-times for release are shown in Table 2 (columns three and four). The data for wild-type ($t_{1/2} = 77$ min) are in good agreement with previous reports (32,33). Release of ADP was faster in the E1197Q mutant ($t_{1/2} = 17$ min) than in wild-type, whereas E552Q ($t_{1/2} = 60$ min) was similar to wild-type. Formation of Vi-trapped ADP was faster in both mutants as compared to wild-type (Table 2, column one) consistent with more potent inhibition of ATPase by Vi (above). Trapped Vi-ADP is thought to mimic a transition state of ATP hydrolysis, therefore one may conclude that the mutations have altered the nature of the transition state, but have not abolished it.

Sequential rounds of Vi trapping of nucleotide. In Figure 4, Vi-trapping was carried out using $[\alpha^{32}\text{P}]\text{ATP}$, and the stoichiometry of trapped ADP was measured ("1st round trapping"). In wild-type and both mutant proteins it was close to 1 mol/mol Pgp. Trapped ADP was then released by extended incubation (wild-type and E552Q, 3 hr; E1197Q, 90 min). DTT, glycerol, and ATP were included in centrifuge column buffers to avoid enzyme denaturation. Stoichiometry of nucleotide remaining bound is shown ("After releasing 1st trap"). A second round of Vi-trapping with $[\alpha^{32}\text{P}]\text{ATP}$ was carried out, and it was seen that the stoichiometry of trapped nucleotide was again 1 mol/mol Pgp. The same results were seen with mutant E1197A. Previously, a similar experiment was reported with human Pgp, using 8-azido-ATP, and it was concluded that Gln mutants differed from wild-type in being unable to undergo a second round of
trapping (31). As shown here, this is not the case when the natural ATP is used with mouse Pgp.

Summarizing this section, the data show that the E552Q and E1197Q mutant mouse Pgp carry out multiple ATPase turnovers, but at much-reduced rate compared to wild-type. Affinity for MgADP is not altered, neither is the rate of release of Vi-trapped ADP slowed, suggesting that deceleration of product ADP release is not the cause of functional impairment. The facts that multiple turnovers of ATPase occur, and that sequential trapping of ADP by Vi is not impaired, suggest that the explanation that the mutations prevent a second ATPase turnover required to reset the drug sites is also unsatisfactory.

2. Effects of Ala, Asp, and Lys mutations at residues Glu552 and Glu1197. To further study the role of the catalytic Glu residues we introduced Ala, Asp, and Lys at positions 552 and 1197 in mouse mdr3 Pgp. ATPase activities of the pure proteins were measured as in Fig. 1, and turnover rates are given in Table 3. In each case activity was linear for 4 hr, as in Fig. 1. Activities of these mutants were very low, and drug stimulation was much reduced. The results, especially with the Asp mutants, reveal a strict requirement for the wild-type Glu carboxyl for normal catalysis. Asymmetry between effects of mutations in N- and C-terminal NBDs was seen to some extent with Lys, but not with Ala and Asp mutants. Apparent $K_m$(MgATP) values were determined (Table 3); again, asymmetry was not apparent in Ala or Asp mutants.
When incubated with Vi and [α-32P]ATP, all of the mutants trapped significant amounts of radioactive nucleotide (Figure 5A). Thin layer chromatography was carried out to determine the nature of the trapped nucleotide. In wild-type there was zero ATP trapped under these experimental conditions, confirming previous data (32,34). Both E552Q and E1197Q behaved similarly to wild-type; however in the other mutants a substantial fraction (26-47%) of the trapped nucleotide was in the form of ATP (data not shown). It should also be mentioned that whereas the amounts of nucleotide retained after centrifuge column elution in absence of Vi were negligible in wild-type, Gln, Asp, and Lys mutants, with E552A and E1197A, Vi-independent retention amounted to 0.4 and 0.15 mol nucleotide /mol Pgp, respectively, of which ~75% was ATP (data not shown). Half-times for release of the Vi-trapped nucleotide are given in Table 2. In all cases, release occurred at a faster rate than in wild-type. Rates of onset of nucleotide-trapping in presence of Vi and [α-32P]ATP were measured at room temperature in order to slow the reaction, and are given in Table 2. This analysis revealed that the Lys mutants trapped nucleotide much more slowly than wild-type, and that the other mutants were generally similar to wild-type.

Figure 5B shows [14C]ADP trapping in presence of Vi for each of the mutants. In all cases trapping of the nucleotide was strongly stimulated by verapamil, showing that none of the mutations affected communication between the catalytic sites and the drug-binding sites. The Gln, Ala and Asp mutants trapped significant amounts of ADP, but the Lys mutants were much less able to do so. If Vi was omitted, there was negligible trapping of ADP in any of the mutants.
Summarizing this section, each of the mutants tested displayed low but real ATPase and ability to trap nucleotide after preincubation with Vi plus ATP. Surprisingly Asp and Lys mutants were not much different in these properties from Gln and Ala mutants. Release of trapped nucleotide was generally faster than in wild-type, the single exception being E552Q. The Lys mutants were less able to trap ADP added directly with Vi and this was consistent with a significantly reduced rate of onset of trapping measured in Lys mutants with Vi plus ATP. Asymmetry between effects of the same mutation placed in either NBD1 or NBD2 was seen in some instances, notably with Gln mutants, but was the exception rather than the rule. In both Ala mutants there was measurable Vi-independent retention of nucleotide, which together with the tendency to retain ATP as well as ADP, was redolent of the behavior of the E552A/E1197A mutant described previously (32).

3. Effects of E552A/S528A and E552A/S1173A mutations. Residues Ser528 and Ser1173 occur in the highly-conserved LSGGQ ABC signature sequences in NBD1 and NBD2 of Pgp, respectively. These Ser residues are known from X-ray crystallography of ABC transporters (12,14) and photocleavage experiments (16) to lie close to the γ-P of ATP bound in catalytic sites. Ser1173 is expected to project into the same ATP-binding site that Glu552 is part of, whereas Ser528 is expected to project into the ATP-binding site that Glu-552 is not part of. S528A and S1173A mutations alone have mild effects, reducing ATPase by only 26% (33). We previously found that the combination mutant S528A/S1173A showed synergistic effects, producing much stronger inhibition of ATPase than was seen with either mutation singly (33), and that the combination
mutant E552A/E1197A showed synergistic effects, producing a conformation of Pgp that was able to tightly-bind ATP and ADP (32). Thus, it was of interest to test effects of the combinations E552A/S528A and E552A/S1173A.

Figure 6B shows ATPase activities of these two purified mutant proteins, compared to E552A in Fig. 6A. Turnover rates are given in Table 3 (last two lines). With E552A/S528A significant turnover was not measurable. Duplicate preparations of pure enzyme gave identical results. This emphasizes that we can in fact detect a zero or very close to zero turnover rate in a mutant Pgp. The rate in E552A/S1173A Pgp was extremely low, similar to that previously seen in E552A/E1197A and lower than in S528A/S1173A. Duplicate purifications of E552A/S1173A gave the same result. Figure 7 shows retention of nucleotide by these mutants after incubation with [α-32P]ATP with or without Vi, then passage through a centrifuge column. With Vi present both mutant proteins retained significant amounts of nucleotide (close to 1 mol/mol Pgp in presence of verapamil); however TLC analysis (not shown) revealed that the retained nucleotide was a mixture of ATP and ADP (E552A/S528A, 48% ATP; E552A/S1173A, 40% ATP) as compared to zero ATP in wild-type3. Also there was considerable retention of nucleotide even in the absence of Vi. Rates of formation of the retained nucleotide species were slower than wild-type in both mutants, whereas nucleotide release was faster (Table 2). Release of retained nucleotide was well-fit by a single exponential curve (not shown).

Both mutant proteins showed trapping of [14C]ADP in presence of Vi (Fig. 8) with E552A/S1173A approaching 1 mol/mol and E552A/S528A lower amounts. Verapamil
increased the level in both cases, showing communication between NBDs and drug-binding sites was intact. In absence of Vi negligible trapping of [1^4C]ADP occurred.

Summarizing this section, combination of S528A or S1173A with the E552A mutation did produce synergistic effects, notably to strongly inhibit ATPase activity, to foster Vi-independent occlusion of nucleotide, and to increase the proportion of ATP vs. ADP occluded.
DISCUSSION

General. Our long-term goal is to understand the molecular mechanism of ATP hydrolysis and its coupling to drug transport in P-glycoprotein (Pgp). In this paper we have studied further the role of two conserved Glu residues (Glu552 and Glu1197 in mouse mdr3 Pgp) that are known to be critical for function in Pgp (28,31) and are thought to function as "catalytic carboxylates" in the ABC transporter family (35). In addition, we combined the mutation E552A at the catalytic carboxylate position in NBD1 with single Ser to Ala mutations at the conserved Ser of the LSGGQ ABC signature motif in either NBD1 or NBD2, to study synergistic interaction of these residues.

Studies of the E552Q and E1197Q mutations. Previous work (28,31) had concluded that these mutations in mouse or human Pgp eliminated ATPase activity. However, here we showed that this is not the case, rather the activity is reduced to a low but significant level. Although the activities reported are low, we are confident that they are not due to contaminants on the following grounds. First duplicate purifications of the same mutant enzyme consistently gave the same activity. Second, a mutant with zero or very close to zero activity was found (see below), also reproducible in duplicate purifications. Third the activity of E552Q and E1197Q mutant proteins showed the same biphasic response to verapamil (activation at lower concentration, inhibition at higher) as seen in wild-type Pgp.

It had previously been proposed (28) that retardation of ADP release could be the cause of loss of ATPase activity in these mutants. Here we evaluated this proposal...
experimentally by measurement of $K_i(MgADP)$ and of rate of release of Vi-trapped ADP. Neither of these experiments reproduces exactly the physiological situation, for example $K_i(MgADP)$ is expected to measure affinity of the "open" NBD dimer, and release of Vi-trapped ADP is much slower than release of ADP in the normal catalytic pathway. On the other hand, if the mutations did cause tenacious retention of product ADP, to the extent necessary to achieve the impairment of ATPase activity seen, then in comparisons of mutant versus wild-type enzymes, one might reasonably expect $K_i(MgADP)$ to be significantly decreased, which did not occur, or the rate of release of Vi-trapped ADP to be significantly slowed, whereas it was actually unchanged (E552Q) or accelerated (E1197Q). Therefore we do not feel that retardation of ADP release provides a satisfactory explanation for the functional impairment in the mutants.

A different explanation for the effects of these mutations was derived from work with human Pgp and 8-azido-ATP in ref. 31, where it was proposed that the mutations prevented a second round of ATPase turnover, required to reset the drug-binding site. However, we found here with mouse E552Q and E1197Q Pgp that multiple turnovers of ATP hydrolysis occurred, and sequential rounds of Vi-ADP trapping could be achieved. Thus, in reference to mouse Pgp, the explanation offered for functional impairment caused by these mutations in ref. 31 appears unsatisfactory.

We did see asymmetric behavior of the two mutations, in that, for example, E552Q had 2.6 times higher ATPase than E1197Q, and $K_m(MgATP)$ was $\geq 5.2$-fold higher. This is in accord with previous findings of asymmetric effects on NBD1 vs NBD2 produced by these mutations (29,30). Both mutants were able to trap Vi-ADP when preincubated with ATP and Vi, consistent with previous observations using 8-azido-ATP.
(28,31). Thus, in contrast to the situation in $F_1$-ATPase (23,24) substitution of the catalytic Glu carboxyl side-chain by Gln did not abolish formation of the transition state complex. The transition state complex of the mutants is not the same as in wild-type however, as evidenced by changed IC$_{50}$ for Vi inhibition and changes seen in rates of formation and release of trapped Vi-ADP. It is also interesting that the turnover rate for the double mutant E552Q/E1197Q was 0.01 s$^{-1}$ (32), which is considerably lower than that of either E552Q or E1197Q (Table 1). Thus presence of one intact NBD moderately improves turnover. Proteolysis studies had shown that Vi-8-azido-ATP trapping occurs in both NBDs (30,31) in the single mutants, thus both NBDs appear to hydrolyse 8-azido-ATP.

Studies of E552A, E1197A, E552D, E1197D, E552K and E1197K mutations.

Substitution by Ala should effectively preclude any orientation or polarization of the attacking water by the residue at this position. Substitution by Asp could retain such properties, but move the water significantly further from the $\gamma$-P of ATP. Substitution by Lys should fail to polarize the attacking water correctly and disrupt charge balance within the catalytic site. It was somewhat surprising therefore that in terms of their effect on ATPase turnover, all six mutants behaved similarly (Table 3), showing a low residual activity. In Ala and Asp mutants, $K_m$(ATP) was reduced, as in Gln mutants (above). The results emphasize the strict requirement for Glu at this position, with Asp failing to provide significant partial activity. It appears that in absence of the Glu side-chain, other residues in the catalytic site take over as (far less efficient) catalytic residues. Possible candidates are Gln471 and Gln1114, the "Q-loop" residues, as originally suggested in
The NBD asymmetry seen in the Gln mutants was not seen with Ala or Asp mutants. Therefore asymmetric effects seen previously (29,30) may be limited to particular mutants and not generalizable to wild-type Pgp.

Overall the effect of the mutations was to change characteristics of the transition state, but not to abolish it. Vi-trapping experiments showed some differences between the mutants in stoichiometry and in rates of formation and release of Vi-trapped nucleotide. In terms of stoichiometry, the mutants behaved somewhat similarly to wild-type, with even Lys mutants trapping significant amounts of Vi-ADP when preincubated with Vi and MgATP, although less so when preincubated with Vi and ADP. In all cases release of Vi-ADP was faster than wild-type (Table 2); rates of formation of trapped Vi-ADP after preincubation with Vi and ATP were more variable. One notable feature was that E552K and E1197K mutants showed slow onset of trapping of Vi-ADP from Vi and MgATP, as if the MgATP association rate were slow, or a conformational change to the closed (dimer) state were impaired by the introduction of positive charge.

As noted in RESULTS, after preincubation with Vi and ATP there was an increase in the proportion of ATP that was retained after centrifuge column elution by Ala, Asp, and Lys mutants. This, together with the Vi-independent retention of nucleotide noted in the Ala mutants, reveals a tendency toward the behavior seen in the E552Q/E1197Q, E552A/E1197A, E552D/E1197D and E552K/E1197Q "double" mutants (32). Our conclusion from that work was that the double mutants arrested the catalytic cycle at a step immediately after ATP binding, with the NBDs in a closed (putative dimeric) state in which nucleotide became occluded. This occluded state could not
proceed efficaciously to the normal transition state. It would seem possible that the single mutations studied here engender the same effect, to lesser degree.

It may be noted that in diverse ABC transporters, various mutations at the catalytic carboxylate (Glu) position have uniformly been seen to reduce ATPase activity to very low levels, examples being Asp, Ala, Cys, Gln and Ser in BmrA (36), Gly in KpsT (37), and Asp in HisP (38), as well as Gln or Ala in numerous other cases. The usual explanation proffered is that the wild-type Glu residue represents the "catalytic base". In contrast to what was found in F$_1$-ATPase (23,24) however, mutations of the catalytic Glu to Gln do not appear to abolish transition state formation in Pgp, at least as measured by Vi-ADP trapping. Our data with Pgp suggest that alterations to the integrity of the transition state, a tendency toward partial arrest in a closed state, and the slow onset of Vi-ADP trapping (in Lys mutants), may be related to a different, common explanation for reduced ATPase turnover, i.e. that the mutations impair rapid formation of an NBD dimer interface that normally occurs upon ATP binding, and/or distort that interface when it does form thus altering characteristics of the closed conformation in which the transition state normally forms. This explanation regarding Pgp may be applicable to other ABC transporters.

Combinations of mutation E552A with S528A or S1173A produce synergistic effects. In X-ray structures of dimeric ABC transporter NBDs, the side-chain -OH of conserved Ser in the LSGGQ signature sequence is close to the $\gamma$-P of bound ATP (12,14,15). Also, bound Vi-ADP induces photocleavage at this position (16). The relevant Ser residues in Pgp are Ser528 (NBD1) and Ser1173 (NBD2). A previous study showed that while
mutation of either Ser to Ala reduced ATPase by 26%, combined mutation S528A/S1173A produced 96.4% inhibition and reduced $k_{cat}/K_m$ by 100-fold, providing clear evidence for a synergistic effect of the combined mutations (33). Also, the single mutations E552A and E1197A produced ATPase turnover rates of 0.075 and 0.066 s$^{-1}$ (Table 3) whereas the combined mutation E552A/E1197A had turnover rate of 0.012 s$^{-1}$ (32). From the X-ray structures it is clear that in the dimeric NBD complex residues Glu552 of NBD1 and Ser1173 of NBD2 would be close to the $\gamma$-P of the same ATP molecule, whereas Glu552 and Ser528 would be close to the $\gamma$-P of two different ATP molecules. Therefore it was interesting to find out what happened when E552A was combined with S528A or S1173A.

Both combinations were strongly synergistic as can be seen from ATPase turnover rates in Table 3. E552A/S1173A had turnover rate of 0.012 s$^{-1}$. In E552A/S528A, turnover rate was zero or close to zero. These results reinforce the idea that structural integrity of the putative NBD dimer interface is critical for function. The combination mutants also evinced other features that seem to be correlated with arrest of the catalytic pathway at the stage of a closed, occluded nucleotide state, namely emergence of Vi-independent sequestration of nucleotide after incubation with Vi plus ATP (Figure 7), and the finding that ATP represents a significant fraction of the occluded nucleotide (see RESULTS).

**Summary.** The role of catalytic carboxylates Glu552 and Glu1197 in Pgp was studied. We found that two previous explanations for the effects of mutations E552Q or E1197Q to inhibit function in Pgp, namely that ADP release is slowed (28) or that a second
(drug-site-resetting) round of ATP hydrolysis is blocked (31) appeared unsatisfactory. Mutation to Gln, Ala, Asp, or Lys altered characteristics of the transition state, but did not prevent its formation, in contrast with mutation of the analogous Glu in F$_1$-ATPase. The effects of the mutations can be interpreted as due to interference with formation of the correct interdigitated NBD dimer interface, which we propose is necessary to generate a closed conformation which follows upon ATP binding and progresses to formation of the normal transition state. Synergistic effects of E552A with either S528A or S1173A were seen, also supporting this latter interpretation.

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REFERENCES

1. Ambudkar, S.V., Dey, S., Hrycyna, C.A., Ramachandra, M., Pastan, I., and Gottesman, M.M. (1999) Ann. Rev. Pharmacol. and Toxicol. 39, 361-398.

2. Gottesman, M.M., Fojo, T., and Bates, S.E. (2002) Nature Rev. Cancer 2, 48-58.

3. Senior, A.E., Al-Shawi, M.K., and Urbatsch, I.L. (1995) FEBS Lett. 377, 285-289.

4. Van Veen, H.W., Margolles, A., Muller, M., Higgins, C.F., and Konings, W.N. (2000) EMBO J. 19, 2503-2514.

5. Sauna, Z.E., Smith, M.M., Muller, M., Kerr, K.M., and Ambudkar, S.V. (2001) J. Bioenerg. Biomembr. 33, 481-491.

6. Urbatsch, I.L., Gimi, K., Wilke-Mounts, S., and Senior, A.E. (2000) J. Biol. Chem. 275, 25031-25038.

7. Urbatsch, I.L., Tyndall, G.A., Tombline, G., and Senior, A.E. (2003) J. Biol. Chem. 278, 23171-23179.

8. Qu, Q., Russell, P.L., and Sharom, F.J. (2003) Biochemistry 42, 1170-1177.

9. Loo, T.W., and Clarke, D.M. (2000) J. Biol. Chem. 275, 19435-19438.

10. Loo, T.W., Bartlett, M.C., and Clarke, D.M. (2002) J. Biol. Chem. 277, 41303-41306.

11. Urbatsch, I.L., Gimi, K., Wilke-Mounts, S., Lerner-Marmarosh, N., Rousseau, M-E., Gros, P., and Senior, A.E. (2001) J. Biol. Chem. 276, 26980-26987.

12. Smith, P.C., Karpowich, N., Millen, L., Moody, J.E., Rosen, J., Thomas, P.J., and Hunt, J.F. (2002) Mol. Cell 10, 139-149.

13. Locher, K.P., Lee, A.T., and Rees, D.C. (2002) Science 296, 1091-1098.
14. Chen, J., Lu, G., Lin, J., Davidson, A.L., and Quiocho, F.A. (2003) Mol. Cell 12, 651-661.
15. Hopfner, K.P., Karcher, A., Shin, D.S., Craig, L., Arthur, L.M., Carney, J.P., and Tainer, J.A. (2000) Cell 101, 789-800.
16. Fetsch, E.E., and Davidson, A.L. (2002) Proc. Natl. Acad. Sci. (USA) 99, 9685-9690.
17. Moody, J.E., Millen, L., Binns, D., Hunt, J.F., and Thomas, P.J. (2002) J. Biol. Chem. 277, 21111-21114.
18. Verdon, G., Albers, S.V., van Oosterwijk, N., Dijkstra, B.W., Driessen, A.J.M., and Thunnissen, A.M.W.H. (2003) J. Mol. Biol. 334, 255-267.
19. Janas, E., Hofacker, M., Chen, M., Gompf, S., van der Does, C., and Tampé, R. (2003) J. Biol. Chem. 278, 26862-26869.
20. Mildvan, A.S. (1997) Proteins, 401-416.
21. Story, R.M., and Steitz, T.A. (1992) Nature 355, 374-376.
22. Abrahams, J.P., Leslie, A.G.W., Lutter, R.E., and Walker, J.E. (1994) Nature 370, 621-628.
23. Nadanaciva, S., Weber, J., and Senior, A.E. (1999) J. Biol. Chem. 274, 7052-7058.
24. Nadanaciva, S., Weber, J., and Senior, A.E. (2000) Biochemistry 39, 9583-9590.
25. Menz, R.I., Walker, J.E., and Leslie, A.G.W. (2001) Cell 106, 331-341.
26. Amano, T., Tozawa, K., Yoshida, M., and Murakami, H. (1994) FEBS Lett. 348, 93-98.
27. Hung, L.W., Wang, I.X., Nikaido, K., Liu, P.Q., Ames, G.F., and Kim, S.H. (1998) Nature 396, 703-707.

28. Urbatsch, I.L., Julien, M., Carrier, I., Rousseau, M.E., Cayrol, R., Gros, P., and Ruysschaert, J.M. (2000) Biochemistry 39, 14138-14149.

29. Vigano, C., Julien, M., Carrier, I., and Gros, P. (2002) J. Biol. Chem. 277, 5008-5016.

30. Carrier, I., Julien, M., and Gros, P. (2003) Biochemistry 42, 12875-12885.

31. Sauna, Z.E., Müller, M., Peng, X.H., and Ambudkar, S.V. (2002) Biochemistry 41, 13989-14000.

32. Tombline, G., Bartholomew, L.A., Urbatsch, I.L., and Senior, A.E. (2004) J. Biol. Chem. 279, in press.

33. Tombline, G., Bartholomew, L.A., Gimi, K., Tyndall, G.A., and Senior, A.E. (2004) J. Biol. Chem. 279, 5363-5373.

34. Sankaran, B., Bhagat, S., and Senior, A.E. (1997) Biochemistry 36, 6847-6853.

35. Geourjon, C., Orelle, C., Steinfels, E., Blanchet, C., Deleage, G., Di Pietro, A., and Jault, J-M. (2001) Trends Biochem. Sci. 26, 539-544.

36. Orelle, C., Dalmas, O., Gros, P., Di Pietro, A., and Jault, J.M. (2003) J. Biol. Chem. 278, 47002-47008.

37. Bliss, J.M., Garon, C.F., and Silver, R.P. (1996) Glycobiology 6, 445-452.

38. Shyamala, V., Baichwal, V., Beall, E., and Ames, G. (1991) J. Biol. Chem. 266, 18714-18719.
**TABLE 1**

ATPase activity, $K_m$(MgATP) and $K_i$(MgADP) values for E552Q and E1197Q mutants.

| Pgp        | ATPase activity $^a$ (s$^{-1}$) | $K_m$(MgATP) (mM) | $K_i$(MgADP) (mM) |
|------------|---------------------------------|-------------------|-------------------|
| Wild-type  | 4.5 (7.0x)                      | 1.3 $^b$          | 0.26              |
| E552Q      | 0.18 (3.9x)                     | 0.47 $^b$         | 0.16              |
| E1197Q     | 0.07 (1.3x)                     | 0.09              | 0.16              |

$^a$ Turnover rate with -fold stimulation by 150 $\mu$M verapamil in brackets. Note a turnover rate of 2.35 s$^{-1}$ corresponds to a specific activity of 1 $\mu$mol ATP hydrolysed/min/mg Pgp. Data are means of quadruplicate determinations.

$^b$ $K_m$ is apparent only, since curves did not saturate (see text and Fig. 2A,B).
TABLE 2

Retention and release of nucleotide by mutant Pgp upon preincubation with Vi and [α-32P]ATP

To assess rate of formation of trapped nucleotide, Pgp was preincubated with Vi and [α-32P]ATP at room temperature then passed through centrifuge columns at varied time. To measure rate of release of trapped nucleotide, preincubation with Vi and [α-32P]ATP was for 20 min at 37°C, then the Pgp was passed through a centrifuge column. Eluates were allowed to incubate for various times at 37°C before passage through a second centrifuge column to determine bound nucleotide.

| Pgp           | Rate of formation of trapped nucleotide (t1/2, min) | Rate of release of trapped nucleotide (min⁻¹) | (t1/2, min) |
|---------------|-----------------------------------------------------|---------------------------------------------|-------------|
| Wild-type     | 0.72                                                | 0.009                                       | 77          |
| E552Q         | 0.35                                                | 0.012                                       | 60          |
| E1197Q        | 0.40                                                | 0.041                                       | 17          |
| E552A         | 0.71                                                | 0.035                                       | 20          |
| E1197A        | 2.95                                                | 0.027                                       | 26          |
| E552D         | 0.24                                                | 0.099                                       | 7           |
| E1197D        | 0.38                                                | 0.139                                       | 5           |
| E552K         | 118                                                 | 0.032                                       | 22          |
| E1197K        | 134                                                 | 0.099                                       | 7           |
| E552A/S528A   | 3.6                                                 | 0.065                                       | 11          |
| E552A/S1173A  | 2.5                                                 | 0.033                                       | 21          |
**TABLE 3**

ATPase activity and $K_m$(MgATP) values for Pgp mutants.

| Pgp           | ATPase activity $^a$ | $K_m$(MgATP) |
|---------------|----------------------|--------------|
|               | (s$^{-1}$)           | (mM)         |
| Wild-type     | 4.5 (7.0x)           | 1.3 $^b$     |
| E552A         | 0.075 (2.0x)         | 0.11         |
| E1197A        | 0.066 (1.3x)         | 0.11         |
| E552D         | 0.075 (1.5x)         | 0.17         |
| E1197D        | 0.061 (1.3x)         | 0.27         |
| E552K         | 0.056 (2.0x)         | 1.2 $^b$     |
| E1197K        | 0.033 (1.1x)         | $\geq$3 $^b$|
| E552A/S528A   | Not measurable $^c$  | Not measurable $^c$ |
| E552A/S1173A | 0.012                | 0.50         |

$^a$ Turnover rate with -fold stimulation by 150 $\mu$M verapamil in brackets. Note a turnover rate of 2.35 s$^{-1}$ corresponds to a specific activity of 1 $\mu$mol ATP hydrolysed/min/mg Pgp.

$^b$ $K_m$ is apparent only, since curves did not saturate.

$^c$ See Fig. 6B data.
LEGENDS TO FIGURES

Figure 1. ATPase activity of E552Q and E1197Q mutant Pgp. \( ^{32}\)Pi release from [\(\gamma^{32}\)P]ATP (3 mM) was measured by charcoal adsorption assay as described in EXPERIMENTAL PROCEDURES. Each assay used 0.5 \(\mu\)g of activated Pgp in an assay volume of 50 \(\mu\)l at 37˚C. ●, with 150 \(\mu\)M verapamil; ○, verapamil absent. Each data point is an average of quadruplicates.

Figure 2. Competitive inhibition of E552Q and E1197Q mutant Pgp ATPase activity by MgADP. Competitive inhibition of Pgp ATPase by MgADP was assayed by measuring \(^{32}\)Pi release from [\(\gamma^{32}\)P]ATP as in Figure 1, using varied concentration of ATP, in presence of zero (●), 250 \(\mu\)M (○), 500 \(\mu\)M (▼), or 1 mM (▲) added ADP. A, wild-type; B, E552Q; C, E1197Q. Each data point is an average of triplicates.

Figure 3. Rate of release of Vi-trapped [\(\alpha^{32}\)P]ADP by E552Q and E1197Q mutants. Release of Vi-trapped [\(\alpha^{32}\)P]ADP from Pgp was measured as described in EXPERIMENTAL PROCEDURES. ●, wild-type; □, E552Q; △, E1197Q. Each point is an average of triplicates.

Figure 4. Sequential rounds of Vi-trapping of [\(\alpha^{32}\)P]ADP by E552Q and E1197Q mutants. Details of the experiment are given in EXPERIMENTAL PROCEDURES. Pgp was first incubated with [\(\alpha^{32}\)P]ATP in presence of Vi, and trapped [\(\alpha^{32}\)P]ADP was determined after centrifuge column elution ("1st round trapping"). This Vi-trapped [\(\alpha^{32}\)P]ADP was
32P]ADP was released by further incubation of the eluates, followed by a second centrifuge column elution. The histograms above "After releasing 1st trap" show the [α-32P]ADP retained at this stage. The Pgp was then incubated again with [α-32P]ATP in presence of Vi, and trapped [α-32P]ADP was determined after centrifuge column elution ("2nd round trapping"). Data are means of quadruplicate experiments.

Figure 5. Stoichiometry of nucleotide retention in catalytic sites of mutant Pgp after preincubation with vanadate and [α-32P]ATP or [8-14C]ADP. A. Activated wild-type and mutant Pgp were incubated for 20 min at 37°C with 200 µM [α-32P]ATP and 200 µM Vi, in the presence or absence of 150 µM verapamil, then passed through a centrifuge column to allow determination of retained nucleotide. For further details see EXPERIMENTAL PROCEDURES. B. As in A except incubation was for 120 min at 37°C with 100 µM [8-14C]ADP. Data are means of at least triplicate experiments.

Figure 6. ATPase activity of E552A, E552A/S528A, and E552A/S1173A mutant Pgp. ATPase assays were conducted as in Figure 1. Verapamil when present was at 150 µM. A. ●, E552A with verapamil; ○, E552A, no verapamil. B. ■, E552A/S1173A with verapamil; ▲, E552A/S528A with verapamil; □, E552A/S1173A, no verapamil. For clarity, E552A/S528A with no verapamil is omitted, the points were on the same line as E552A/S1173A, no verapamil. Data are means of quadruplicate experiments.

Figure 7. Stoichiometry of nucleotide retention in catalytic sites of mutant Pgp after preincubation with [α-32P]ATP with or without vanadate. The experiment was carried
out as in Figure 5A, except that vanadate was either absent or present. When present verapamil was 150 \( \mu \text{M} \). Data are means of at least triplicate experiments.

Figure 8.  **Stoichiometry of nucleotide retention in catalytic sites of mutant Pgp after preincubation with vanadate and [8-\( ^{14} \text{C} \)]ADP.** The experiment was carried out as in Figure 5B. When present verapamil was 150 \( \mu \text{M} \). Data are means of at least triplicate experiments.
FIG. 1
FIG. 2

(A) Graph showing ATP hydrolyzed (μmol/min/mg) vs. ATP (μM).

(B) Graph showing ATP hydrolyzed (μmol/min/mg) vs. ATP (μM).

(C) Graph showing ATP hydrolyzed (μmol/min/mg) vs. ATP (μM).
FIG. 4
FIG. 7
Properties of P-glycoprotein with mutations in the "catalytic carboxylate" glutamate residues
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