Role of Carboxylate Residues Adjacent to the Conserved Core Walker B Motifs in the Catalytic Cycle of Multidrug Resistance Protein 1 (ABCC1)*

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MRP1 belongs to subfamily “C” of the ABC transporter superfamily. The nucleotide-binding domains (NBDs) of the C family members are relatively divergent compared with many ABC proteins. They also differ in their ability to bind and hydrolyze ATP. In MRP1, NBD1 binds ATP with high affinity, whereas NBD2 is hydrolytically more active. Furthermore, ATP binding and/or hydrolysis by NBD2 of MRP1, but not NBD1, is required for MRP1 to shift from a high to low affinity substrate binding state. Little is known of the structural basis for these functional differences. One minor structural difference between NBDs is the presence of Asp COOH-terminal to the conserved core Walker B motif in NBD1, rather than the more commonly found Gln present in NBD2. We show that the presence of Asp or Gln following the Walker B motif profoundly affects the ability of the NBDs to bind, hydrolyze, and release nucleotide. An Asp to Gln mutation in NBD1 enhances its hydrolytic capacity and affinity for ADP but markedly decreases transport activity. In contrast, mutations that eliminate the negative charge of the Asp side chain have little effect. The decrease in transport caused by the Asp to Gln mutation is associated with an inability of MRP1 to shift from high to low affinity substrate binding states. In contrast, mutation of Gln to Asp markedly increases the affinity of NBD2 for ATP while decreasing its ability to hydrolyze ATP and to release ADP. This mutation eliminates transport activity but potentiates the conversion from a high to low affinity binding state in the presence of nucleotide. These observations are discussed in the context of catalytic models proposed for MRP1 and other ABC drug transport proteins.

ATP-binding cassette (ABC) transporters are ubiquitous transmembrane proteins that couple ATP hydrolysis to the energy-dependent transport of a wide variety of endogenous and exogenous molecules across biological membranes. Multidrug resistance protein (MRP) 1 (ABCC1) belongs to the “C” subfamily of the ABC superfamily and was discovered by virtue of its ability to cause multidrug resistance when overexpressed in a human small cell lung cancer cell line (1, 2). The MRP1 multidrug resistance phenotype is similar to that resulting from overexpression of P-glycoprotein (P-GP), and involves resistance to many relatively hydrophobic, natural product type, cytotoxic agents. However, unlike P-GP, MRP1 can also transport various structurally unrelated organic anionic conjugates, including: glutathione, glucuronide, and sulfate conjugates, such as the potent mediator of inflammation, cysteiny1 leukotriene LTC4, the cholestatic glucuronide-conjugated estrogen E17G, the sulfate conjugate estrone 3-sulfate, and the glutathione epoxide conjugate of the highly mutagenic aflatoxin B1 (3–10).

Typically, the functional form of ABC proteins consists of two hydrophilic nucleotide-binding domains (NBDs) located at the cytoplasmic surface of the membrane and two hydrophobic transmembrane spanning domains (MSD) that are thought to form the translocation pathway. The predicted topology of some of the ABCCC transporters such as MRP1, MRP2, MRP3, MRP6, and MRP7, as well as SUR1 and SUR2, is unusual in that it includes an additional NH2-terminal MSD that probably contains five transmembrane segments and has an extracellular NH2 terminus (11–13).

The NBDs of ABC proteins contain three conserved motifs required for nucleotide binding and hydrolysis: the Walker A and B motifs (14) and the ABC signature sequence (LSGGQ) or C-motif (15). ATP hydrolysis and substrate transport is strongly dependent on cooperativity between the NBDs. In P-GP, inactivation of either NBD by mutation of essential residues in the Walker A motif completely abolishes ATP-dependent transport activity (16, 17). The NBDs of P-GP can also be exchanged without loss of function and in the original model proposed for the catalytic cycle of P-GP (18), the alternating hydrolysis of one ATP molecule by either NBD results in transport of one molecule of substrate (19, 20). More recently, a variation of this model has been proposed in which one ATP hydrolysis event results in substrate transport and hydrolysis of a second ATP is required to restore the protein to a high affinity substrate binding state (21). Whether or not each NBD is limited to a distinct role in the transport process has not been established. However, studies of P-GP in which positions of NBDs were exchanged suggest that the location of the NBD in the protein may influence its ability to bind and hydrolyze nucleotide. Thus although interchangeable without loss of function, the NBDs may not be functionally identical in the intact transporter (18, 22). Recent biochemical evidence in the

This paper is available on line at http://www.jbc.org

Received for publication, June 2, 2003, and in revised form, July 18, 2003
Published, JBC Papers in Press, July 27, 2003, DOI 10.1074/jbc.M305786200

* This work was supported the National Cancer Institute of Canada with funds from the Terry Fox Run and the Medical Research Council of Canada Grant MT-10519 and a post-doctoral fellowship from La Ligue Nationale Contre le Cancer (France) (to L. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: ABC, ATP-binding cassette; MRP, multidrug resistance protein; LTC4, leukotriene C4; E17G, 17β-estradiol-17-β-(glucuronide); NBD, nucleotide-binding domain; MSD, membrane-spanning domain; Mt, ATP-γS, adenosine 5’-O-(thio-triphosphate); AMP-PNP, adenosine 5’-(β,γ-imino)triphosphate; P-GP, P-glycoprotein; mAb, monoclonal antibody.

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E. coli histidine permease suggests that its two identical HisP subunits may be functionally asymmetric (23).

Compared with many transporters, the NBDs of ABCC proteins are structurally relatively divergent such that there is greater similarity between comparable NBDs in different ABCC members than between NBDs in the same protein (24). A considerable body of evidence also indicates that NBDs within a single protein differ functionally. We and others have shown that in MRP1, the NBDs differ considerably with respect to ATP binding and vanadate-dependent trapping of ADP (24–28). Furthermore, although trapping of ADP by NBD2 of MRP1 requires that NBD1 be able to bind and possibly to hydrolyze ATP, the binding of ATP by NBD1 is preserved when NBD2 is inactivated by essential mutations in its Walker A motif (24, 25). Inactivation of each NBD also has different effects on transport activity. A number of NBD1 mutations that eliminate ATP hydrolysis and diminish ATP binding decrease transport activity by only 60–70%, whereas comparable NBD2 mutations inactivate the protein (24). The different effects of these mutations strongly suggest that the catalytic cycle of MRP1 differs from that first proposed for P-GP and prokaryotic transporters with two essentially identical NBDs (16, 29).

To begin defining the structural basis of the functional differences between the two NBDs of MRP1, we have examined the role of the acidic residue immediately COOH-proximal to the highly conserved core of its Walker B motif. In NBD1, this residue is Asp, whereas in NBD2, it is Glu, as is found in the NBDs of most ABC proteins (Fig. 1). The function of this Glu residue had been studied in several ABC transporters, such as HisP and P-GP, but its role in the catalytic cycle remains controversial. In HisP, mutation of this residue to Gln results in a protein able to bind ATP but with no detectable ATPase activity (30, 31). Comparable mutations in NBD1 and NBD2 of the P-GP murine homologue, mdr3, result in loss of ATPase activity but the protein can trap ADP at the mutated NBD in the presence of vanadate, suggesting that ADP release is impaired (32). In contrast, similar studies of human P-GP found that although ADP release was impaired when the corresponding Glu residues in both NBDs were mutated to Gln, single mutants in either NBD were able to release ADP but unable to proceed through a second catalytic cycle (33).

To investigate the roles played by Asp793 and Glu1455 adjacent to the core Walker B motif, we have both exchanged these residues and mutated them to a number of amino acids lacking carboxylate side chains. The mutations have been expressed singly and in combination using a baculovirus dual expression system we have described previously, which enables highly efficient reconstitution of a functional transporter from two "half-molecules" that can be readily separated by SDS-PAGE (34). Thus quantitative effects of the mutations on nucleotide binding, trapping, and release by individual NBDs can be easily assessed (24). Similarly, the influence of mutations on the ability of the high affinity MRP1 substrate, LTC4, to photolabel sites in the second and third MSDs can also be determined (35). These studies revealed that differences between the ATP binding and hydrolysis characteristics of the two NBDs of MRP1 are attributable in large part to the presence of Asp rather than Glu adjacent to
the NBD1 Walker B motif at position 793. In addition, we demonstrate that contrary to expectations based on the alternating site model of catalysis, increasing the hydrolytic activity of NBD1 by introduction of Glu in place of Asp decreases rather than increases transport of LTC4. This decrease in transport activity is associated with an increase in ADP trapping at the mutant NBD1 and a marked reduction in trapping by the cytoplasmic domain of NBD2. Finally, we present evidence that conversion of the protein from a high affinity substrate binding conformation to a low affinity transition state occurs when NBD2 is occupied by either ATP or ADP and is prevented when NBD1 is occupied by ADP.

EXPERIMENTAL PROCEDURES

Materials—8-Azido-[α-32P]ATP, 8-azido-[γ-32P]P-ATP, and 8-azido-[α-32P]ADP were purchased from Affinity Labeling Technologies, Inc. (Lexington, KY). Orthovanadate, ATP, AMP-PNP, ADP, and ATP-γ-S compounds were from Sigma.

MRP1 cDNA Mutation—The pFBDual-MRP1 1–932/932-1531 construct (pFBDual-halves, pFBDual-D123/D45) was cloned into pFAST-BAC Dual (Invitrogen). This construct, encoding the N2- and COOH-proximal half-molecules of MRP1, has been described by Gao et al. (24, 34).

8-Azido-Glu793 and Asp455 mutants were generated by site-directed mutagenesis using a Clontech transformer kit. The templates used for site-directed mutagenesis, pGEM-NBD1 and pGEM-NBD2, were described previously (24). The forward primers for D793E and E1455D—

| Sequence | Description |
|----------|-------------|
| 5′-CCTCTTCGATGAGCCCCTCTCAGC-3′ | 8-Azido-[γ-32P]P-ATP | 8-Azido-[α-32P]ATP | 8-Azido-[α-32P]ADP | 8-Azido-[γ-32P]ATP | 8-Azido-[α-32P]ATP | 8-Azido-[α-32P]ADP |

were used to replace the equivalent region of pFBDual-Asp45 to give pFB-Dual-D45/E1455D. This was then digested with Ncol and Kpn1 and the Ncol-Kpn1 fragment was used to replace the equivalent region of pFBDual-Asp45 to give pFB-Dual-D45/E1455D. Finally, the SalI-XbaI fragment of pFBDual-halves was isolated and cloned into pFBDual-D45/E1455D, which had been digested with the same enzymes, to generate pFB-Dual-D45/E1455D. In the D793E/E1455D double mutant, the SalI/XbaI fragment with the mutation in NBD1 was isolated and ligated to pFBDual-D45/E1455D, which had been digested with the same enzymes, to generate pFBDual-halves/D793E/E1455D.

Other mutations (Gln793, Ser793, Asn793, Gln1455, Ser1455, Asn1455, and Leu45) were carried out in the pFBDual-halves vector using the Quikchange Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Oligonucleotides used for site-directed mutagenesis were synthesized by ACGT Corp. The forward primers for D793Q, D793N, D793S, E1455Q, E1455N, E1455S, and Leu45 were—

| Sequence | Description |
|----------|-------------|
| 5′-CCTCTTCGATGAGCCCCTCTCAGC-3′ | 3′-CCTCTTCGATGAGCCCCTCTCAGC-3′ | 3′-CCTCTTCGATGAGCCCCTCTCAGC-3′ | 3′-CCTCTTCGATGAGCCCCTCTCAGC-3′ | 3′-CCTCTTCGATGAGCCCCTCTCAGC-3′ | 3′-CCTCTTCGATGAGCCCCTCTCAGC-3′ | 3′-CCTCTTCGATGAGCCCCTCTCAGC-3′ |

Unless otherwise indicated in the figure legend, insect membrane vesicles (75 μg of protein) were resuspended in transport buffer (50 μM Tris-HCl, pH 7.4, 250 μM sucrose, and 0.02% of NaN3) containing 5 mM MgCl2 and 5 μM 8-azido-[γ-32P]P-ATP or 8-azido-[α-32P]ATP (ALT Corp.; specific activity between 5 and 20 Ci mmol−1).

After 5 min in a 96-well plate, the membranes were irradiated for 7 min on ice in a Stratalinker UV cross-linker (λ = 302 nm). After addition of 150 μM of ice-cold buffer (50 μM Tris-HCl, pH 7.4, 0.1 mM EDTA, and 5 mM MgCl2), the membranes were centrifuged at 14,000 rpm for 15 min at 4 °C. A second wash was performed and the pellets were resuspended in 14 μl of ice-cold buffer. After addition of Laemmli buffer (4×) containing diethiothreitol (100 mM final), membrane vesicles were electrophoresed on gradient SDS-PAGE (7–15%). After drying for 2 h at 80 °C, gels were autoradiographed in a PhosphorImager (Amer sham Biosciences) and autoradiographed using Kodak Bio-Max MS films.

Vanadate-induced Trapping of 8-Azido-[γ-32P]ATP by MRP1—The 100 μM vanadate solution was prepared by dissolving 184 μg of sodium orthovanadate in 9.35 ml of water. The pH of vanadate solution was adjusted to 10 using 2 N HCl and boiled for 10 min. The solution must become colorless. Just before use, this solution must be boiled 10 min and diluted to 10 μl with transport buffer.

Membrane vesicles (20 μg of protein) were resuspended in transport buffer containing 5 mM MgCl2 and 15–15 μM 8-azido-[γ-32P]P-nucleotide (ALT Corp.; specific activity between 5 and 20 Ci mmol−1). Unless otherwise indicated in the figure legend, the 15-min incubation at 37 °C was performed in the presence or absence of 1 μM vanadate. The reaction was started by addition of 8-azido-[γ-32P]P-ATP, 8-azido-[α-32P]ATP, or 8-azido-[α-32P]ADP and stopped by transfer on ice and addition of ice-cold buffer as previously described.

Unreacted nucleotides were then removed (2×) by addition of 150 μM of ice-cold buffer followed by centrifugation. Pellets were resuspended in 14 μl of ice-cold buffer and irradiated for 7 min on ice in a Stratalinker UV cross-linker (λ = 302 nm) as previously described by Gao et al. (24). After the addition of Laemmli buffer (4×), containing diethiothreitol (100 mM final), membrane vesicles were electrophoresed on gradient SDS-PAGE (7–15%). After drying for 2 h at 80 °C, gels were autoradiographed in a PhosphorImager (Amersham Biosciences) and autoradiographed using Kodak Bio-Max MS films.

Transport of [3H]HILTCγ into Insect Membrane Vesicles—Uptake of [3H]HILTCγ (50 nm, 182 Ci mmol−1, PerkinElmer Life Sciences) into membrane vesicles was measured at 23 °C in the presence of ATP (4 μM) or AMP (4 μM) using a rapid filtration technique as previously described (7).

Photoaffinity Labeling by MRP1 with [3H]HILTCγ—Unless otherwise indicated in the figure legend, insect membrane vesicles (75 μg of protein in 20 μl) were incubated with [3H]HILTCγ (0.13 μCi, 200 nm) at room temperature for 20 min. Cells were then frozen in liquid nitrogen (1 min), followed by UV irradiation (1 min). This was repeated 10 times. Membrane vesicles were resuspended in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% 2-mercaptoethanol, 10% glycerol) and were fixed by 25% isopropyl alcohol, 65% water, and 10% acetic acid for 30 min, and gels were dried at 80 °C for 2 h and autoradiographed on Kodak Bio-Max MS films (35).

RESULTS

Wild-type and various mutant MRP1 half-molecules were co-expressed in Sf21 cells infected with baculovirus dual-expression vectors. The levels of co-expressed MRP1 fragments in membrane vesicles from infected cells were then determined by immunoblotting, using the rat mAb MRP1p (epitope amino-
acids 238–247) and murine mAb MRPm6 (epitope amino-acids 1511–1520) to detect NH$_2$- and COOH-proximal fragments, respectively. Membrane vesicles from insect cells infected with a β-Gus baculovirus construct were used as a negative control. No MRP1 specific binding was detected by mAb MRPr1 and mAb MRPm6 in cells infected with the β-Gus vector.

**Effect of D793E and E1455D Single and Double Mutations on LTC$_4$ Transport Activity—**LTC$_4$ uptake by membrane vesicles from Sf21 cells expressing wild-type and mutant MRP1 half-molecules was determined at 23 °C, as described by Loe et al. (7). The expression levels of mutant and wild-type proteins were determined to be comparable by immunoblotting (Fig. 2A) and transport activity values have not been normalized. Uptake at 3 min, by vesicles containing the D793E mutant NH$_2$-terminal fragment and the wild-type COOH-proximal half was ~20% of that obtained with vesicles containing two wild-type fragments. The transport activity of vesicles containing either the E1455D mutant fragment and the wild-type NH$_2$-terminal half, or co-expressed D793E and E1455D mutant fragments was similar to that of the β-Gus control (Fig. 2B). Thus the effects of the D793E and E1455D mutations on LTC$_4$ transport were similar to previously described mutations of the Walker A motif that eliminate ATP hydrolysis and decrease ATP binding by NBD1 and NBD2, respectively (24, 25).

**Photolabeling with 8-Azido-[γ-32P]ATP in the Presence of AMP-PNP at 4 °C—**To determine whether or not the D793E and E1455D mutations altered ATP binding, studies were carried out at 4 °C using the radioactive photoactivable analog of ATP, 8-azido-[γ-32P]ATP, hydrolysis of which results in loss of the γ-$^32$P label (24, 33). As previously reported (24, 25), ATP binding by wild-type MRP1 occurred preferentially at NBD1. In addition, like SUR1 (40), this ATP labeling was strongly competed by 1 mM AMP-PNP, a non-hydrolyzable ATP analog (Fig. 2C). ATP labeling of wild-type MRP1, D793E, and E1455D mutant proteins was similar and occurred preferentially at NBD1, whereas it was slightly decreased at NBD1 of the double mutant protein. However, ATP labeling by wild-type and mutant proteins differed at NBD2. Labeling at NBD2 in the E1455D and D793E/E1455D mutant proteins was strongly and moderately enhanced, respectively, relative to the wild-type NBDs. In all cases, AMP-PNP strongly competed 8-azido-[γ-$^32$P]ATP labeling at both NBDs of mutant proteins, as observed with the co-expressed wild-type fragments.

**Nucleotide Trapping by Wild-type MRP1 and D793E, E1455D Single and Double Mutant Proteins Using 8-Azido-[α-$^32$P]ATP—**To determine whether ATP hydrolysis by the mutant NBDs was altered, ADP trapping experiments were performed at 37 °C using various concentrations of 8-azido-[α-$^32$P]ATP in the presence or absence of vanadate. In these experiments, free and weakly bound nucleotides were removed by centrifugation and washing before membrane vesicles were UV cross-linked. As previously reported, ADP trapping by the wild-type NBDs was highly vanadate-dependent and occurred preferentially at the NBD2 (Fig. 3A). In the presence of vanadate, weak trapping by the wild-type NBD2 was observed using 2.5 μM 8-azido-[α-$^32$P]ATP and this was strong at 5 and 15 μM (Fig. 3A). At the highest concentration, weak photolabeling of the wild-type NBD2 could also be detected in the absence of vanadate (Fig. 3A). In comparison to wild-type MRP1, ADP trapping was increased at the D793E mutant NBD1 and decreased at the co-expressed wild-type NBD2. Increased labeling of the mutant NBD1 was observed at all 8-azido-[α-$^32$P]ATP concentrations tested and unlike the wild-type NBD1, was readily detectable at 2.5 μM 8-azido-[α-$^32$P]ATP with both the D793E and D793E/E1455D mutants (Fig. 3, A and C). In addition, photolabeling was detected at all concentrations from 2.5 to 15 μM 8-azido-[α-$^32$P]ATP in the absence of vanadate, indicative of high affinity binding of nucleotide even in the absence of stabilization by vanadate (Fig. 3, A and C).
Catalytic Cycle of MRP1

The E1455D mutation dramatically increased photolabeling of the mutant NBD2 and unlike the D793E mutation, increased rather than decreased labeling of the co-expressed wild-type NBD1. Labeling of the mutant NBD2 was only modestly enhanced in the presence of vanadate and was detectable at 8-azido-[α-32P]ATP concentrations as low as 1 μM (Fig. 3B). Similarly, labeling of the co-expressed wild-type NBD1 was also only weakly vanadate-dependent and could be detected in the absence of 2.5 μM 8-azido-[α-32P]ATP. Despite the striking increase in photolabeling of the E1455D mutant NBD2, in the double D793E/E1455D mutant, labeling occurred predominantly at NBD1 and was markedly diminished at NBD2 relative to the E1455D single mutant. Thus the labeling profile of the double mutant is the reverse of that observed with the wild-type protein, but unlike the wild-type, labeling of the double mutant is the reverse of that observed with the E1455D mutant NBD2.

Photolabeling with 8-Azido-[γ-32P]ATP or 8-Azido-[α-32P]ADP at 37 °C—The relatively weak vanadate dependence of photolabeling observed when using 8-azido-[α-32P]ATP, particularly with the E1455D and D793E/E1455D mutants, raised the possibility that the mutant NBD2 may be capable of tight binding of both ATP and ADP. To examine this possibility, the trapping experiments were repeated using 8-azido-[γ-32P]ATP. In the presence of 1 mM vanadate, photolabeling of wild-type and the D793E mutant NBD1 by 8-azido-[γ-32P]ATP was barely detectable, presumably as a result of the hydrolytic loss of the [γ-32P]PO4 (Fig. 4A). These data combined with trapping using 8-azido-[α-32P]ADP indicate that the nucleotide “trapped” by the NBDs of the wild-type and D793E mutant proteins was indeed ADP. In contrast, both NBDs of the E1455D mutant were strongly labeled by 8-azido-[γ-32P]ATP, while in the D793E/E1455D double mutant labeling of NBD2 was much reduced and labeling of NBD1 was essentially eliminated (Fig. 4A). In contrast to the ATP binding observed at 4 °C, photolabeling at 37 °C of the E1455D mutant NBD2 was stronger than the co-expressed wild-type NBD1 and was only slightly competed by 1 mM AMP-PNP. These experiments suggest that the tight binding of ATP by the E1455D mutant NBD2 stimulates the binding of ATP by the co-expressed wild-type NBD1 and conversely, that the increased trapping of ADP at the D793E mutant NBD1, diminishes ATP binding by both the co-expressed wild-type and E1455D mutant NBD2.
To directly examine ADP binding and trapping by wild-type and mutant proteins, we carried out experiments similar to those described above using two concentrations of 8-azido-[α-32P]ADP. Membrane vesicles were incubated with 8-azido-[α-32P]ADP (5 and 15 μM) and washed prior to cross-linking (Fig. 4B). In the presence of vanadate, ADP binding increased in a concentration-dependent manner at NBD2 in membranes containing co-expressed wild-type half-molecules (Fig. 4B, lanes 2 and 3). Photolabeling was barely detectable at 5 μM 8-azido-[α-32P]ADP but was strongly increased at 15 μM. Very little binding was observed at the wild-type NBD1 even at the higher concentration of nucleotide. In comparison to wild-type, ADP labeling of the D793E mutant NBD1 was increased and was almost maximal using 5 μM 8-azido-[α-32P]ADP (Fig. 4B, lanes 2, 4, and 5). In contrast, photolabeling by 15 μM 8-azido-[α-32P]ADP was clearly decreased at the wild-type NBD2 co-expressed with the D793E mutant NBD1, when compared with the co-expressed wild-type half-molecules (Fig. 4B, lanes 2–5).

ADP binding by the E1455D single mutant was strongly increased at both NBDs relative to the co-expressed wild-type fragments although the majority of photolabeling occurred at the mutant NBD2. In the D793E/E1455D double mutant, photolabeling of NBD2 was diminished and labeling of NBD1 was increased relative to the single D793E and E1455D single mutants, so that ADP labeling was similar at both NBDs (Fig. 4B, lanes 2–9). Thus, as observed with 8-azido-[γ-32P]ATP, the increased binding of ADP by the E1455D mutant NBD2 appears to stimulate tight binding of ADP at both the wild-type and D793E mutant NBD1. In contrast, the increased ADP binding of the D793E mutant NBD1 decreases ADP binding by both the wild-type and E1455D mutant NBD2, as would be predicted by an alternating site model of catalysis.

**Evaluation of ADP Release by Wild-type and D793E, E1455D Single and Double Mutant Proteins**—It has been demonstrated that ADP release constitutes the rate-limiting step in the normal catalytic cycle of P-GP (41). In addition, recent studies with murine mdr3 indicate that mutation of the Glu residue following the Walker B core of either NBD to Gln permitted ATP hydrolysis but decreased or prevented ADP release (32). Similarly, mutation of the Glu residue following the Walker B core of either NBD to Gln permitted ATP hydrolysis but decreased or prevented ADP release (33). Consequently, we investigated the influence of D793E, E1455D, and D793E/E1455D MRP1 mutants on ADP release by each NBD. Membrane vesicles were first incubated for 15 min at 37 °C with 5 μM 8-azido-[α-32P]ATP in the absence of vanadate. Unbound α-32P-nucleotides were removed by washing. Membranes were then either cross-linked immediately, or reincubated at 37 °C for 15 min in the absence of nucleotide in a buffer containing MgCl2, prior to cross-linking. No photolabeling of the wild-type co-expressed half-molecules was observed, even in samples cross-linked immediately following washing (Fig. 5, lane 2). As described above, weak photolabeling of NBD1 in samples cross-linked immediately after washing could be detected with the D793E and D793E/E1455D mutants (Fig. 5, lanes 3 and 5). However, labeling was lost when the samples were reincubated prior to cross-linking (Fig. 5, lanes 9 and 11). In contrast, the E1455D mutation displayed very strong labeling of the mutant NBD2 that persisted following the reincubation period (Fig. 5, lanes 4 and 10). In addition, the co-expressed wild-type NBD1 also retained a readily detectable level of labeling following reincubation indicating that nucleotide release by this NBD was also affected. Unlike the single E1455D mutant, very little photolabeling of NBD2 was retained following reincubation of the D793E/E1455D double mutant (Fig. 5, lanes 5 and 11).

To further investigate the impairment of ADP release by the E1455D and the D793E/E1455D mutants, cold ADP (1 mM) was added 3 min before the end of the initial nucleotide labeling with 8-azido-[α-32P]ATP at 37 °C in the absence of vanadate at 37 °C for 15 min. In lanes 6–8, 3 min before the end of the incubation, 1 μM cold ADP was added. In lanes 9–11, after removal of unincorporated nucleotides by centrifugation (2×) in transport buffer, proteins were incubated in transport buffer containing MgCl2, at 37 °C for 15 min. After removal of unincorporated nucleotide by centrifugation, the samples were cross-linked and separated on gradient gels. Similar results were obtained in at least three additional independent experiments. The position of the labeled MRP1 NH2-half and COOH-half polypeptides are indicated, and endogenous proteins labeled are indicated by an arrow. 

**LTC₄ Photolabeling by Wild-type and D793E, E1455D Single and Double Mutant Proteins Under Hydrolytic and Non-hydrolytic Conditions**—We have shown previously that LTC₄ can photolabel MRP1 at sites in MSD2 and MSD3 and that photolabeling, particularly of the site in MSD2, markedly slows down nucleotide release at NBD1, whereas the latter increases nucleotide binding and markedly slows down nucleotide release at NBD2. As found previously using co-expressed wild-type half-molecules, in the absence of nucleotide LTC₄ predominantly photolabels a site in the NH2-proximal half of the protein and to a lesser extent a site in the COOH-proximal fragment (Fig. 6A, 6C).
transition to a low affinity substrate binding state.

The LTC\textsubscript{4} labeling profile of the E1455D mutant in the absence of nucleotide was also similar to that observed for wild-type MRP1 (Fig. 6B, lanes 2 and 11). However, this mutation potentiated the effect of nucleotide on LTC\textsubscript{4} binding (Fig. 6B, lanes 1, 3, and 4). Unlike the co-expressed wild-type half-molecules, ATP\&S, ATP, and ATP plus vanadate were similarly effective in decreasing LTC\textsubscript{4} photolabeling of the major NH\textsubscript{2}-proximal site. These results are consistent with the proposal that the occupancy of NBD2 by either ATP or ADP can result in a shift from the high affinity to low affinity substrate binding conformation.

The double D793E/E1455D mutant behaved in a manner very similar to that of the D793E single mutation, with the exception that ATP\&S retained some ability to diminish LTC\textsubscript{4} labeling (Fig. 6B, lanes 6–11). Thus these results together with those of vanadate trapping experiments are again consistent with the suggestion that increased trapping of ADP by the D793E mutant NBD1 decreases the nucleotide binding and hydrolysis by wild-type and E1455D mutant NBD2 and prevents conversion to a low affinity transition state.

Effect of Substituting Asp\textsuperscript{793} and Glu\textsuperscript{1455} with Non-carboxylic Amino Acids on LTC\textsubscript{4} Transport, Nucleotide Binding, and ADP Trapping—The results of experiments described above indicate that the introduction of Asp in place of Glu in NBD2 of MRP1 is sufficient to cause at least some alterations in nucleotide binding and hydrolysis that are similar to those observed when the carboxylate side chain of the comparable Glu residues in P-GP and mdr3 were eliminated (32, 33). To determine whether there may be additional consequences to eliminating the carboxylate side chain, we mutated Asp\textsuperscript{793} and Glu\textsuperscript{1455} to Asn, Gln, and Ser as observed in cystic fibrosis transmembrane conductance regulator. In addition, Glu\textsuperscript{1455} was mutated to Leu. The effects of these mutations on LTC\textsubscript{4} transport and nucleotide binding and trapping were then determined.

Based on densitometry, levels of expression for mutant (Gln\textsuperscript{793}, Ser\textsuperscript{793}, Asn\textsuperscript{793}, Ser\textsuperscript{1455}, Asn\textsuperscript{1455}, and Leu\textsuperscript{1455}) and wild-type fragment were similar (Fig. 7A). The exception was the NH\textsubscript{2}-proximal of the E1455Q mutant that was expressed at a level 1.75 times higher than its COOH-proximal half (Fig. 7A). Because the ATP-dependent uptake of LTC\textsubscript{4} relies upon association of both halves of the protein and the levels of all COOH-proximal fragments were comparable, no adjustment was made to compensate for the increased expression of the NH\textsubscript{2}-proximal half of E1455Q (Fig. 7A). LTC\textsubscript{4} transport activity of each mutant co-expressed with the relevant wild-type half-molecule was then determined and compared with that of the co-expressed wild-type fragments. Unlike the D793E mutation, which decreased LTC\textsubscript{4} transport activity by 80%, NBD1 mutations (D793S, D793N, and D793Q) had little effect on ATP-dependent LTC\textsubscript{4} uptake (Fig. 7B). In contrast, the NBD2 mutations (E1455S, E1455N, E1455Q, and E1455L) like E1455D completely abolished LTC\textsubscript{4} transport (Fig. 7B). Thus with respect to LTC\textsubscript{4} transport, there is no requirement for an acidic residue following the Walker B motif of NBD1, whereas there is a specific requirement for Glu at this location in NBD2.

At 4 °C, mutation of Asp\textsuperscript{793} to Ser, Gln, or Asn decreased photolabeling of NBD1 by 8-azido-[\alpha-\textsuperscript{32}P]ATP to a similar extent. However, NBD1 was still more strongly photolabeled than NBD2, as observed with the wild-type co-expressed halves and, unlike the D793E mutation that diminished labeling of NBD2, labeling was unaffected by substitution with the three polar uncharged residues (Fig. 6A, lanes 3, 5, and 7). The effects of the Glu\textsuperscript{1455} mutations were more variable. Mutation to Ser, Gln, and Leu essentially eliminated labeling of NBD2, whereas labeling of the E1455N mutation was similar to that of the

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**Fig. 6. Effect of ATP\&S or ADP trapping on [\textsuperscript{3}H]LTC\textsubscript{4} photolabeling by wild-type MRP1, D793E, E1455D, and D793E/E1455D mutant proteins.** Wild-type MRP1, D793E (A) and E1455D, D793E/E1455D (B) membrane proteins (75 μg) were incubated in transport buffer containing 5 mM MgCl\textsubscript{2} at 23 °C for 20 min in the absence (−) or presence (+) of ATP (1 mM), vanadate (1 mM), or ATP\&S (4 mM), alone or in combination, prior to addition of [\textsuperscript{3}H]LTC\textsubscript{4} (200 nM, 0.13 μCi). The [\textsuperscript{3}H]LTC\textsubscript{4} photolabeling was performed as described under “Experimental Procedures.” Similar results were obtained in at least three additional independent experiments.
co-expressed wild-type halves of the protein. Labeling of the co-expressed wild-type NBD1 was also slightly decreased by the Ser, Asn, and Leu mutations of Glu1455 (Fig. 8A, lanes 4, 6, 8, and 9). Labeling of the wild-type NBD1 co-expressed with the E1455Q mutation appears to be increased, but we cannot completely exclude the possibility that this is attributable at least in part to the 1.75-fold higher level of expression of this fragment. However, we have shown previously that the free NH₂-terminal half binds ATP much less efficiently than when associated with the other half of the protein.

The effect of the non-conservative mutations on nucleotide binding and ADP trapping was examined at 37 °C using 5 μM 8-azido-[α-32P]ATP in the presence or absence of vanadate, exactly as described for the D793E and E1455Q mutations. Similar to wild-type, in the absence of vanadate, D793S, D793Q, and D793N mutant proteins did not display any nucleotide binding at either NBD (Fig. 8B). This contrasts with the D793E mutation in which photolabeling of NBD1 was readily detectable in the presence of 5 μM 8-azido-[α-32P]ATP and in the absence of vanadate. In the presence of vanadate, the labeling profile of the D793S mutation was very similar to wild-type with nucleotide being bound relatively strongly by NBD2 (Fig. 8B, lanes 3 and 5). Although labeling at NBD2 was decreased in the D793Q and D793N mutants, this domain remained the predominant site of photolabeling (Fig. 8B, lanes 7 and 9).

Like the E1455D mutant, the E1455S, E1455N, and E1455L mutations resulted in strong vanadate-independent photolabeling of NBD2 and increased vanadate-dependent photolabeling of NBD1 (Fig. 8C, lanes 3, 4, and 7–10). The E1455Q
The position of the labeled MRP1 NH2-half and COOH-half polypeptides are indicated, and endogenous proteins labeled are indicated by arrows. Wild-type MRP1 and mutant proteins (D793Q, D793N, D793S, E1455S, E1455Q, E1455N, and E1455L) was evaluated. Membrane vesicles (20 μg) were incubated with 5 μM 8-azido-[α-32P]ATP for 5 min on ice in transport buffer containing 5 mM MgCl2. The samples were photocross-linked and unincorporated nucleotides were removed as described under “Experimental Procedures.” The position of the labeled MRP1 NH2-half and COOH-half polypeptides are indicated, and endogenous proteins labeled are indicated by arrows. A, at 4 °C, 8-azido-[α-32P]ATP photolabeling by wild-type MRP1 and mutant proteins (D793Q, D793N, D793S, E1455S, E1455Q, E1455N, and E1455L) was evaluated. Membrane vesicles (20 μg) were incubated with 5 μM 8-azido-[α-32P]ATP for 5 min on ice in transport buffer containing 5 mM MgCl2. The samples were photocross-linked and unincorporated nucleotides were removed as described under “Experimental Procedures.” Similar results were obtained in at least two independent experiments. The position of the labeled MRP1 NH2-half and COOH-half polypeptides are indicated, and endogenous proteins labeled are indicated by arrows. B and C, at 37 °C under trapping conditions, 8-azido-[α-32P]ADP trapping by wild-type MRP1 mutant proteins (D793Q, D793N, D793S, E1455S, E1455Q, E1455N, and E1455L) was studied. Membrane vesicles (20 μg) were incubated with 5 μM 8-azido-[α-32P]ATP in the absence (−) or presence (+) of 1 mM vanadate for 15 min at 37 °C in transport buffer containing 5 mM MgCl2. After removal of unreacted nucleotide by centrifugation as described under “Experimental Procedures,” the samples were photocross-linked and separated on gradient gels. Similar results were obtained in at least two independent experiments. The position of the labeled MRP1 NH2-half and COOH-half polypeptides are indicated, and endogenous proteins labeled are indicated by arrows.

**DISCUSSION**

A characteristic feature of the NBDs of ABCC proteins is the lack of a typical Glu residue COOH-proximal to the Walker B core motif in NBD1. In the MRP1 and SUR, this residue is Asp and in cystic fibrosis transmembrane conductance regulator it is Ser, whereas in NBD2 of all ABCC proteins it is Glu (Fig. 1). Given the importance of this Glu residue in the catalytic cycle of other ABC transporters, we investigated to what extent the difference between Glu and Asp at this position might contribute to the observed functional differences between the two NBDs of MRP1.

Based on the behavior of NBD2 of MRP1 and other NBDs containing Glu adjacent to the Walker B motif, we anticipated that the D793E mutation in NBD1 would increase ATP hydrolysis and possibly decrease the affinity for ATP. If so, the alternating site model of transport proposed for ABC proteins would predict that such a gain of function mutation should increase substrate transport if hydrolysis at NBD1 was a rate-limiting step, or have no effect in the less likely event that the rate-limiting step involved hydrolysis at NBD2 (21, 29). Contrary to expectation, the mutation decreased transport of LTC4 by ~80% (Fig. 2B). However, consistent with the prediction that the mutation would increase the hydrolytic activity of NBD1, it resulted in a major increase in the trapping of ADP (Fig. 3A). Furthermore, the affinity for ADP was enhanced, as indicated by an increased labeling of the mutant NBD1 with 8-azido-[α-32P]ADP at 37 °C (Fig. 4B, lanes 4 and 5). In contrast to the wild-type NBD1, vanadate did not further increase nucleotide trapping by the D793E mutant when photolabeling was carried out with 8-azido-[α-32P]ATP at 37 °C (Fig. 3A). Similar results were observed when beryllium was used rather than vanadate (data not shown), indicating that the ADP-NBD1 complex formed is relatively stable in the absence of either agent. In addition, the D793E mutation slightly decreased binding of 8-azido-[γ-32P]ATP at 4 °C by the co-expressed wild-type NBD2 (Fig. 2C) and caused a major decrease in the trapping of ADP at 37 °C in the presence of vanadate (Fig. 3A). We have demonstrated previously that binding and/or hydrolysis of ATP by NBD2 is required for MRP1 to shift from a high to low affinity substrate binding state (35, 42). Consistent with the decrease in ATP binding and ADP trapping at the co-expressed wild-type NBD2, the decrease in LTC4 binding observed with the wild-type protein in the presence of ATP and vanadate was completely abrogated by the D793E mutation (Fig. 6A, lanes 7–10). Thus the D793E mutation decreases LTC4 transport by preventing the transition from a high to low affinity binding state.

The E1455D mutation essentially eliminated transport of LTC4 as observed previously with mutations that inactivate NBD2 (Fig. 2B). However, unlike such mutations (24, 25), it increased binding of 8-azido-[γ-32P]ATP by the mutant NBD at low concentrations of nucleotide (Fig. 2C) and dramatically increased vanadate-independent photolabeling at 37 °C (Fig. 3B). The relative vanadate independence of binding at 37 °C suggested that the nucleotide tightly bound or trapped by the mutant NBD2 in the presence of 8-azido-[α-32P]ATP could be ATP or a mixture of ATP and ADP (Fig. 3, B and C). The latter possibility was supported by experiments using 8-azido-[γ-32P]ATP (Fig. 4A) or 8-azido-[α-32P]ADP (Fig. 4B) in place of 8-azido-[α-32P]ATP. Strong photolabeling of the mutant NBD2 was observed with both nucleotide analogs. Binding in the absence of vanadate was also sufficiently stable to withstand thorough washing to remove free nucleotide prior to photocross-linking. Thus the E1455D mutation substantially increased affinity for both ATP and ADP. The strong labeling observed with 8-azido-[γ-32P]ATP at 37 °C also indicates that
the rate of hydrolysis is substantially decreased relative to wild-type NBD2.

The E1455D mutation also strongly enhanced nucleotide binding and vanadate-dependent trapping at the co-expressed wild-type NBD1 (Fig. 3, B and C). Previously, we and others have provided evidence that ATP binding by NBD1 stimulates binding and hydrolysis of ATP by NBD2 (26, 27). Results presented here are consistent with a reciprocal stimulation of ATP binding and hydrolysis by NBD1 when NBD2 is occupied by ATP. However, the NBDs differ with respect to the consequences of occupancy with ADP. The “classical” alternating sites model of catalysis predicts that both NBDs cannot be occupied by ADP at the same time. Thus mutations that increase occupancy of one NBD by ADP would be expected to decrease occupancy of the other. Consistent with such a prediction, the D793E mutation increased ADP trapping at the mutant NBD1 and decreased vanadate-dependent trapping by the co-expressed wild-type NBD2. Contrary to expectation, the markedly increased 8-azido-⎡ATP binding by the E1455D mutant NBD2 was accompanied by increased rather than decreased ADP photolabeling of the co-expressed wild-type NBD1, suggesting that both NBDs could be simultaneously occupied by ADP. This increase in labeling was only modestly vanadate-dependent (Fig. 3B). Consequently, it remains possible that the two NBDs are trapped in different ADP-bound conformational states. We also found that the E1455D mutation drastically decreased nucleotide release at 37°C, as evidenced by an inability to elute the two NBDs that were photolabeled with 8-azido-⎡ATP (Fig. 5) or 8-azido-⎡ATP (data not shown) with a large excess of cold nucleotide. In addition, binding persisted following washing and reincubation for 15 min in the presence of MgCl2 and absence of free nucleotide (Fig. 5).

In the absence of nucleotides, LTC4 photolabeling was unaffected by the E1455D mutation. However, the ability of ATPγS and ATP to decrease LTC4 binding was clearly potentiated relative to wild-type MRP1 (Fig. 6B). Furthermore, unlike wild-type MRP1, the effect of ATPγS and ATP to decrease LTC4 binding was clearly potentiated relative to wild-type MRP1 (Fig. 6B). In addition, ATPγS was much more effective at reducing binding by the E1455D mutant when compared with the wild-type protein. This strongly suggests that the shift to a low affinity state occurs when NBD2 binds ATP and persists as long as the NBD is occupied by either ATP or ADP. Thus the loss of LTC4 transport activity appears to be attributable to the impaired ability of the E1455D mutant to release nucleotide from NBD2 and to re-establish a high affinity binding site.

Like the single mutations, the D793E/E1455D mutation had no effect on LTC4 binding in the absence of nucleotides. However, in the presence of ATP or ATP plus vanadate, the D793E mutation in the double mutant abrogated the shift from a high to a low affinity binding state, despite the potentiating effect observed with the E1455D single mutation. Paradoxically, a decrease in LTC4 binding by the D793E/E1455D double mutant protein was still observed in the presence of ATPγS (Fig. 6B). This may be explained by the fact that in the double mutant, the rate of ATP hydrolysis and extent of ADP trapping at NBD1 is increased while the rate of ATP hydrolysis at NBD2 is markedly decreased. As a consequence, in the presence of ATP and vanadate, NBD1 and NBD2 would be loaded with ADP and ATP, respectively. With the poorly hydrolyzable ATPγS, the predominant form of the protein would be expected to be one in which both NBDs are occupied by the analog. Thus overall, the data support a mechanism in which the transition from high to low affinity states is prevented by occupancy of NBD1 with ADP and is triggered by the binding of ATP to NBD2, which in turn is stimulated by ATP binding to NBD1.

We also examined the effects of several mutations of Asp793 and Glu1455 that eliminated the negatively charged carboxylate side chain. Mutation of Asp793 to Asn, Gln, and also Ser, which is the residue found at the comparable position of cystic fibrosis transmembrane conductance regulator, all decreased slightly LTC4 transport activity, whereas the comparable mutations of Glu1455 inactivated the protein (Fig. 7B). These effects of the Asp793 mutations are consistent with a previous report that mutation of this residue to Leu had no effect on transport activity (49). The Asp793 mutations also caused a slight decrease in ATP binding at both NBD1 and NBD2 relative to the wild-type protein, suggesting that the negative charge on the carboxylate residue might make a weak contribution to ATP binding (Fig. 8A).

Like the E1455D mutation, the substitution of Glu1455 with Gln, Asn, Leu, and Ser completely abolished LTC4 transport activity (Fig. 7B). However, in contrast to the E1455D mutation, which at 4°C increased ATP binding at NBD2, binding was decreased by the Gln, Ser, and Leu mutations, whereas the Asn mutation had little or no effect relative to wild-type (Fig. 8A, lanes 4, 6, 8, and 9). This suggests that at 4°C, the longer Asp and Asn side chains enhance ATP binding, which in the case of Asn, appears to compensate for the loss of the negative charge from the Glu residue in the wild-type protein. At 37°C, Ser, Asn, and Leu mutations of Glu1455 strongly increased nucleotide binding and trapping at both NBDs in the presence and absence of vanadate, as observed with the E1455D mutation, whereas binding by the Gln mutant protein, although increased relative to wild-type MRP1, remained strongly vanadate-dependent (Fig. 8C). This suggests that the Glu mutant, unlike the other mutant proteins including the E1455D mutant, is able to hydrolyze ATP and probably release ADP in the absence of vanadate. This observation may explain differences observed between murine mdr3 and human P-GP in which the comparable Glu residues had been mutated (32, 33). In the case of mdr3, replacement of the Walker B proximal Glu residue of one NBD by non-carboxylic amino acids has been reported to prevent ADP release, whereas in P-GP such mutations appear to permit release but prevent progression through a subsequent step in the catalytic cycle. Thus the behavior of the MRP1 Glu1455 to Glu mutation is similar to that of the comparable inactivating mutation in human P-GP, whereas the other mutations appear to block ADP release, as reported in studies of murine mdr3 (32, 33).

In the case of both cystic fibrosis transmembrane conductance regulator and SUR1, it has been suggested that NBD1 binds but does not hydrolyze ATP (44, 45). In wild-type MRP1, vanadate-dependent ADP trapping is only very weakly detectable at NBD1 (24). However, with all Glu1455 mutants examined, we consistently observed a strong increase in vanadate-dependent trapping of ADP by the co-expressed wild-type NBD1 indicating that this NBD is competent to hydrolyze ATP and to trap ADP. Furthermore, the occupancy of NBD1 by ADP appears to prevent entry into a low affinity transition state. Whether ATP hydrolysis by NBD1 is obligatorily required to reset the protein in a high affinity binding state following substrate transport, or whether release of ATP will suffice is not firmly established. However, a requirement for such a hydrolytic step, as envisaged in an alternating sites model of transport, is very difficult to reconcile with the strong negative effect on transport of mutations that enhance ATPase activity of NBD1, such as the D793E mutation, and the relatively minor effect of mutations at the same location that eliminate the negative side chain at the position of Asp793.

Catalytic Cycle of MRP1
Acknowledgments—We thank our colleagues Drs. Caroline Grant and Gwenaelle Conseil for helpful discussions and advice. The excellent technical assistance from Monika Vasa and Ruth Burth-Wright are appreciated.

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