Membrane transporters of the adenine nucleotide binding cassette (ABC) superfamily utilize two either identical or homologous nucleotide binding domains (NBDs). Although the hydrolysis of ATP by these domains is believed to drive transport of solute, it is unknown why two rather than a single NBD is required. In the well studied P-glycoprotein multidrug transporter, the two appear to be functionally equivalent, and a strongly supported model proposes that ATP hydrolysis occurs alternately at each NBD (Senior, A. E., al-Shawi, M. K., and Urbatsch, I. L. (1995) FEBS Lett 377, 285–289). To assess how applicable this model may be to other ABC transporters, we have examined adenine nucleotide interactions with the multidrug resistance protein, MRP1, a member of a different ABC family that transports conjugated organic anions and in which sequences of the two NBDs are much less similar than in P-glycoprotein. Photoaffinity labeling experiments with 8-azido-ATP, which strongly supports transport revealed ATP binding exclusively at NBD1 and ADP trapping predominantly at NBD2. Despite this apparent asymmetry in the two domains, they are entirely interdependent as substitution of key lysine residues in the Walker A motif of either impaired both ATP binding and ADP trapping. Furthermore, the interaction of ADP at NBD2 appears to allosterically enhance the binding of ATP at NBD1. Glutathione, which supports drug transport by the protein, does not enhance ATP binding but stimulates the trapping of ADP. Thus MRP1 may employ a more complex mechanism of coupling ATP utilization to the export of agents from cells than P-glycoprotein.

**Experimental Procedures**

Materials—8-Azido-[γ-32P]ATP and 8-azido-[α-32P]ATP were purchased from ICM. Sodium orthovanadate, doxorubicin, reduced glutathione, ADP, and MgCl₂ were purchased from Sigma. The Stratational UV Crosslinker 2400 model (wavelength 254-nm) and QuikChange Site Directed Mutagenesis Kit were from Stratagene.

**In Vitro Mutagenesis of MRP1 cDNA**—We have employed the coding sequence of human MRP1 cDNA in the pNUT expression vector (11). The lysine residues at positions 684 (Walker A motif in NBD1, AAG to CTG) and 1333 (Walker A motif in NBD2, AAG to CTG) were mutated to leucine residues by using the QuikChange Site Directed Mutagenesis Kit. The aspartic acid residues at positions 792 and 793 (Walker B motif in NBD1, GAT to CTT) and at 1454 and glutamic acid at 1455 (Walker B motif in NBD2, GAT to CTT and GAG to CTG) were also mutated to leucine residues. To ensure that no other mutations were introduced into the cDNA during mutagenesis, fragments covering the mutations were sequenced completely and used to replace their counterparts in the wild-type MRP1 cDNA in pNUT. The mutations were verified after insertion into the pNUT expression vector.

**Cell Culture and Stable Transfection of MRP1 in BHK Cells**—Baby hamster kidney (BHK-21) cells were cultured at 37°C in 5% CO₂. Stable cell lines expressing wild-type and mutant MRP1s, K684L, K1333L, were generated by using procedures described previously (11).
Membrane and Vesicle Preparations—MRP1-containing membranes were prepared as follows. Cells were grown to confluence in roller bottles and washed once with 20 ml of phosphate-buffered saline. The cells were detached by incubation at 37 °C for 5–10 min with 20 ml of citrate saline. Cells were collected by centrifugation at 4,000 × g for 15 min. The cell pellet was washed once with membrane purification buffer (10 mM Tris-HCl, pH 7.5, 250 mM sucrose, and 0.2 mM MgCl₂). The cells were resuspended in membrane preparation buffer with 1 × protease inhibitors (2 μg/ml apropin, 121 μg/ml benzamidine, 3.5 μg/ml E64, 1 μg/ml leupeptin, and 50 μg/ml Pefabloc) and equilibrated on ice for 20 min at 800 g in a Parr N₂ cavitation bomb. After release of the pressure, the cell homogenate was adjusted to 1 mM in EDTA. The homogenate was then diluted 5-fold with 10 mM Tris-HCl and 25 mM sucrose, pH 7.5, and centrifuged at 1,000 × g for 15 min. The supernatant was overlaid on a 35% sucrose solution containing 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA and centrifuged at 16,000 × g for 30 min. The interface was collected, diluted 5-fold with a solution containing 10 mM Tris-HCl, pH 7.5, 0.1 mM EGTA and 1 × protease inhibitors. Membrane vesicles for drug transport experiments were prepared exactly the same way except the pellet was resuspended in a solution containing 10 mM Tris-HCl, pH 7.5, 250 mM sucrose, and protease inhibitors and passed through a Liposofast™ vesicle extruder (200 nm filter, Avestin, Ottawa, Canada).

Vanadate Preparation and Photofluorophores—vanadate to the membrane vesicles was assayed by a rapid filtration method. The vanadate solution was boiled for 10 min before use. Vanadate solution was prepared by dissolving 184 mg of sodium orthovanadate in 9.35 ml of water and then adding 0.65 ml of 2 M HCl. The samples were then transferred to ice and diluted with 400 μl of ice-cold Tris-EGTA buffer (0.1 mM EGTA and 40 mM Tris-HCl, pH 7.5). The membranes were pelleted in a microcentrifuge in the cold room (4 °C), washed again with 400 μl of ice-cold Tris-EGTA buffer, resuspended in 10 μl of Tris-EGTA buffer, placed on ice and irradiated for 2 min in a Stratalinker UV Crosslinker (λ = 254 nm). The labeled proteins were separated on a polyacrylamide gel (7%). The amount of radioactivity incorporated into MRP1 protein was determined by electronic autoradiography using a Packard Instant Imager, and all of the quantitative evaluations of the results expressed were based on these determinations.

Membrane Vesicle Transport—ATP-dependent transport of [H³H]leukotriene C₄ into the membrane vesicles was assayed by a rapid filtration technique (1, 5). The assays were performed with 3 μg of membrane protein in a 30-μl reaction volume containing 50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 10 mM MgCl₂, 200 mM LTC₄ (17.54 μCi of [H³H]LTC₄) and 4 mM ATP (or as indicated in Fig. 1 legend). After incubation at 37 °C for the periods indicated, the samples were diluted with 1 ml of ice-cold ATPase buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, and 10 mM MgCl₂), and filtered through a nitrocellulose membrane (0.2 μm) that had been equilibrated with 1 × transport buffer. The filter was then washed with 10 ml of cold 1 × transport buffer, air-dried, and placed in 10 ml of biodegradable counting scintillant (Amershams Pharmacia Biotech). The radioactivity bound to the nitrocellulose membrane was determined by liquid scintillation counting (Beckman LS 6000SC).

RESULTS

Photolabeling of MRP1 with N₃[α-³²P] or N₃[γ-³²P]ATP—MRP1 is an exporter of a variety of conjugated hydrophobic substrates (1–7) and has been shown to have ATPase activity that is stimulated by these substrates (11, 12). It is assumed that the NBDs of this ABC protein are responsible for its ability to hydrolyze ATP, but the mechanism whereby these two domains act is unknown. Previous studies of ATP hydrolysis by purified MRP1 and its sensitivity to ADP implicated separate binding sites for ATP and ADP in the protein (19). To evaluate the interactions of the two nucleotides with the NBDs of MRP1, photolabeling experiments have been performed using α-³²P- and γ-³²P-labeled 8-azido-ATP. Fig. 1A shows that labeling with N₃[α-³²P]ATP is dependent on the divalent cations, Mg²⁺ or Mn²⁺. Fig. 1B demonstrates that the labeling is greatly enhanced by increasing concentrations of vanadate, consistent with the trapping of an [α-³²P]ADP-vanadate complex after hydrolysis of MgATP or MnATP (14, 20). In fact there was only a trace amount of labeling in the absence of vanadate. This small amount might reflect the binding of intact N₃[α-³²P]ATP. If this was the case, labeling with N₃[γ-³²P]ATP should occur and it did (Fig. 1C). Surprisingly, however, this labeling was also strongly stimulated by vanadate. This of course cannot reflect binding of N₃ADP-vanadate, since it is not radioactively labeled after release of the γ-³²P label during the hydrolysis step. Instead this labeling must be due to either binding of the whole N₃ATP molecule or phosphorylation. To explain the possible possibility, i.e. that vanadate stimulates the binding of the intact N₃ATP, it is necessary to postulate that vanadate-induced trapping of N₃ADP, a hydrolysis product of N₃[γ-³²P]ATP, at one NBD enhances binding of the latter compound at the other NBD. That is to say vanadate trapping of N₃ADP at one NBD, generated by hydrolysis of N₃ATP, allosterically stimulates the binding of N₃[γ-³²P]ATP to the other NBD. Identification of MRP1 fragments produced by limited proteolysis using site-specific monoclonal antibodies after photolabeling enables determination of which of the NBDs plays each of these roles (see Fig. 4).

Since the amount of labeling with α-³²P-labeled N₃ATP could be due to both N₃ATP binding and N₃ADP trapping, whereas γ-³²P-labeled N₃ATP measures only binding of the triphosphate, the amount of photolabeling by N₃[γ-³²P]ATP should be much less than that by N₃[α-³²P]ATP under the same conditions. Fig. 1D shows that this is indeed observed. This indicates that any phosphorylation of the protein that may contribute to the labeling by N₃[γ-³²P]ATP is substantially less than the amount of N₃[α-³²P]ATP trapped. The actual contribution of phosphorylation as well as the sites of binding of N₃ADP and N₃ATP, their interactions, and the influence of MRP1 transport substrates were investigated in subsequent experiments.

To normalize the use of 8-azido-ATP to probe functionally relevant nucleotide interactions with MRP1, it was necessary to determine how well it supported transport compared with ATP. Fig. 1E shows that if anything the derivatized nucleoside triphosphate supported the uptake of LTC₄ by everted membrane vesicles even better than ATP. In each case, the Kᵢₐ was of the order of 50 μM, a value similar to that found by others using just ATP (5).

Influence of Transport Substrates on Photolabeling—To examine the impact of agents involved in ATP-dependent transport by MRP1 on these implied separate binding sites, the effects of increasing concentrations of doxorubicin (Fig. 2A) and glutathione (Fig. 2B), which is required for the transport of the drug by MRP1, on photolabeling by N₃[α-³²P] and N₃[γ-³²P]ATP were tested. There was only a minor enhancement of N₃[α-³²P]ATP labeling at low doxorubicin concentrations (1 and 10 μM) followed by inhibition at a higher concentration (100 μM). Such a biphasic response was not seen with N₃[γ-³²P]ATP labeling, although 10 and 100 μM concentrations caused inhibition. Hence the drug has slightly different effects on the two azido-ATPs, possibly because of different affinities of the two NBDs for them. The mild stimulation of labeling by N₃[α-³²P]ATP is similar to the increased ATP hydrolysis by purified MRP1 elicited by low concentrations of this drug (11), consistent with the need for hydrolysis for the ADP trapping reflected in the N₃[α-³²P]ATP labeling.

Reduced glutathione had a more striking biphasic effect on N₃[α-³²P]ATP labeling (Fig. 2B) with lower concentrations causing greater than a 2-fold elevation, while high concentrations inhibited almost completely. In contrast, N₃[γ-³²P]ATP labeling was much less sensitive to reduced glutathione with concentrations up to 8 mM causing only a mild stimulation.

Allosteric Interactions between the Two NBDs of MRP1

Photolabeling of MRP1 with N₃[α-³²P] or N₃[γ-³²P]ATP—MRP1 has been performed using a-³²P- and γ-³²P-labeled 8-azido-ATP. Fig. 1A shows that labeling with N₃[α-³²P]ATP is dependent on the divalent cations, Mg²⁺ or Mn²⁺. Fig. 1B demonstrates that the labeling is greatly enhanced by increasing concentrations of vanadate, consistent
Glutathione had a much greater influence on labeling by N3-ADP than by N3-Azo. Experiments were performed in triplicate as described under “Experimental Procedures” (6-min incubations) and S.D. are shown at each point.

Allosteric Interactions between the Two NBDs of MRP1

Before strong but incomplete inhibition at 10 mM, hence glutathione had a much greater influence on labeling by N3-[α-32P]ATP than by N3-[γ-32P]ATP, emphasizing that incorporation of radiolabeling by photolabeling with the differently labeled azido-nucleotides probably reflects different binding events.

Influence of ADP on Photolabeling—Since we had observed previously that low concentrations of ADP accelerated ATP hydrolysis by MRP1, while higher concentrations inhibited (19), an impact of the nucleoside 5′-diphosphate on the photolabeling reactions might be expected. As seen in Fig. 3, labeling with N3-[α-32P]ATP was unaffected by ADP concentrations less than approximately 5 μM and inhibited at higher concentrations consistent with competition for N3-ADP trapping. In striking contrast, labeling with N3-[γ-32P]ATP was progressively increased by ADP concentrations up to 5 μM, where nearly a 5-fold elevation occurred. Clearly, this large stimulatory effect...
by an ADP concentration equal to that of N3ATP must be due to action at a site with much higher affinity for the nucleoside diphosphate than for the triphosphate and is reminiscent of the effect of the nucleoside 5'-diphosphate on hydrolysis (19). Much higher concentrations of ADP were required before this stimulatory effect was overcome to yield a net inhibition, probably due to direct competition for N3ATP binding. It seems reasonable to conclude that the promotion of N3ATP binding observed after photolabeling with N3\[^{32}P\]ATP definitely occurs at NBD2 and possibly also at NBD1. If this is the case, then the strong stimulation of labeling with N3\[^{32}P\]ATP (occurring at NBD1) by low concentrations of ADP (binding at NBD2) should reflect a positive allosteric interaction between the two NBDs.

To further test the suggestion that N3\[^{32}P\]ADP may be trapped at both NBDs but to a lesser extent at NBD1 than NBD2, the effects of ADP on the labeling of the tryptic fragments containing each of the domains was studied (Fig. 4D). When ADP was added to the reaction mixture the labeling of the whole MRP1 protein (188-kDa band) by N3\[^{32}P\]ATP was decreased approximately 20–25% (at 5 \muM ADP, lane 5 versus lane 1 and at 10 \muM ADP, lane 9 versus lane 1). However, labeling of the 65-kDa fragment containing NBD2 was decreased much more, approximately 50–60% (at 5 \muM ADP, lane 7 versus lane 3 and at 10 \muM ADP, lane 11 versus lane 3), whereas that of the 119- and 53-kDa fragments reflecting NBD1 decreased only approximately 10–20% at 5 and 10 \muM ADP. This indicates a much stronger inhibition by ADP of labeling of NBD2 than NBD1 and is consistent with the interpretation that there is preferential binding of ADP to NBD2 and weaker binding to NBD1 (the exclusive site of intact N3ATP binding).

The incorporation of \[^{32}P\] radioactivity into MRP1 on incubation with N\[^{32}P\]ATP could occur by a phosphorylation reaction as well as by the binding of the intact nucleoside triphosphate. Indeed while there was no labeling by N\[^{32}P\]ATP without UV irradiation (Fig. 4E, lane 1) there was labeling by N\[^{32}P\]ATP (Fig. 4E, lane 2). Addition of ADP increased this labeling by less than 2-fold (Fig. 4E, lanes 3–5). UV irradiation

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*Footnote:* X.-b. Chang and J. R. Riordan, unpublished data.
Fig. 4. Localization of sites of photolabeling. A, schematic diagram of MRP1 protein to indicate locations of antibody binding sites and trypsin digestion. The diagram shows that there are three transmembrane domains and two nucleotide binding domains in the MRP1 molecule. The epitope of antibody 42.4 is a decapeptide (residues 723–732) located between the Walker A and B motifs of NBD1 (mAb1). The epitope of antibody 897.2 has been localized only to the large region between residues 1248 and 1531, which covers the entire NBD2 (mAb2). The approximate sites of two trypsin cleavages (TS1 and TS2), which can be deduced, are also indicated. B, Western blots following photolabeling and trypsinolysis. To determine whether α-32P- or γ-32P-labeled 8-azido-ATP bound preferentially to NBD1 or NBD2, the same samples were used to do Western blots and autoradiography (C). 36 μg of MRP1-containing membrane proteins was incubated in 20 μl of the same incubation mixture as in previous figures (lanes 1–6 with 5 μM N3[γ-32P]ATP; lanes 7–12 with 5 μM N3[α-32P]ATP). 5 μM ADP was added to the incubation mixture with N3[γ-32P]ATP to increase the amount bound. After removal from unbound nucleotide and UV irradiation, the samples were then digested in 30 μl of 40 mM Tris-HCl, pH 7.4, 1 mM EDTA for 10 min at room temperature with varying amounts of trypsin. One-third of each sample was then electrophoresed for Western blotting. Fragments in lanes 1–6 were detected by probing with monoclonal antibody 897.2 (recognizing NBD2 fragments). Those in lanes 7–12 were probed with antibody 42.4 (recognizing NBD1 fragments). Trypsin to membrane protein mass ratios were: 0 (lanes 1 and 7); 1:80 (lanes 2 and 8); 1:40 (lanes 3 and 9); 1:20 (lanes 4 and 10); 1:10 (lanes 5 and 11); 1:5 (lanes 6 and 12). Arrows indicate the major trypsin digestion products detected by each of the antibodies. C, autoradiograms following photolabeling and trypsinolysis. Two-thirds of the samples from B were subjected to electrophoresis and autoradiography. Samples in lanes 1–6 were labeled with N3[α-32P]ATP and samples in lanes 7–12 with N3[γ-32P]ATP. N3[γ-32P]ATP labels exclusively the 155- and 65-kDa NBD2-containing bands. N3[α-32P]ATP does not label the latter at all, rather the four major bands detected by the NBD1-specific antibody. D, effects of ADP on labeling of NBD1- and NBD2-containing fragments by 8-azido-[α-32P]ATP. 36 μg of MRP1 membrane was incubated for 10 min at 37 °C in a 20 μl solution containing 40 μM N3[γ-32P]ATP, 0.1 mM EGTA, 10 mM MgCl2, 800 μM vanadate, 5 μM 8-azido-[α-32P]ATP (2 μCi) without ADP (lanes 1–4), with 5 μM ADP (lanes 5–8), or 10 μM ADP (lanes 9–12). Lanes 1, 5, and 9, no trypsin digestion. Lanes 2, 6, and 10, digestion with 1.8 μg of trypsin (1:20) at 23 °C for 10 min. Lanes 3, 7, and 11, 3.6 μg of trypsin (1:10). Lanes 4, 8, and 12, 7.2 μg of trypsin (1:5). E, 8-azido-[γ-32P]ATP labeling with or without UV irradiation. 18 μg of MRP1-containing membrane protein was incubated for 10 min at 37 °C in 10 μl of the same solution used in previous experiments, 5 μM 8-azido-[α-32P]ATP (lane 1) or 8-azido-[γ-32P]ATP (lanes 2–9) without ADP (lanes 1, 2, and 6), 1 μM ADP (lanes 3 and 7), 5 μM ADP (lanes 4 and 8), and 10 μM ADP (lanes 5 and 9). After removing the unbound ATP, the samples in lanes 1–5 were loaded onto the gel without UV irradiation. The samples in lanes 6–9 were UV-irradiated for 2 min at 254 nm and then loaded.

This irradiation-dependent labeling was increased approximately 4-fold by the addition of 10 μM ADP (Fig. 4E, lane 9 versus lane 2), again confirming the observations of Fig. 3B. Thus while some of the radioactivity incorporated into MRP1 to promote photolabeling by the intact substrate augmented incorporation of radioactivity substantially (Fig. 4E, lane 6 versus lane 2), presumably reflecting the binding of N3[γ-32P]ATP to NBD1 revealed in the trypsinolysis experiments.

Promote photolabeling by the intact substrate augmented incorporation of radioactivity substantially (Fig. 4E, lane 6 versus lane 2), presumably reflecting the binding of N3[γ-32P]ATP to NBD1 revealed in the trypsinolysis experiments.
Interdependence of Nucleotide Binding Domains—If ATP is preferentially bound at NBD1 and ADP can be trapped at NBD2, then mutations within the Walker motifs of NBD1 and NBD2 should influence photolabeling by N₃[γ-³²P]ATP and N₃[α-³²P]ATP. The Walker A lysines and Walker B aspartates in the NBDs were mutated as indicated in Fig. 5A. Each of these variants is expressed as fully mature glycoproteins like the wild-type in the presence of AMP (open diamonds) instead of ATP is also indicated.

Although most N₃ADP trapping is at NBD2, the function of NBD2, the predominant site of trapping of the product of ATP hydrolysis, seems also to be more critically required for transport.

DISCUSSION

Since the genes for ABC proteins constitute the largest superfamily in the genomes analyzed to date, understanding their structure and function is of great importance. Their fundamental unit consists of a nucleotide binding domain and a transmembrane domain containing multiple membrane spanning segments and the extramembranous loops joining them. In the exporter families of eukaryotes, two such units are fused to form large membrane proteins, some of which contain additional domains as well. N-terminal of the two fundamental units of members of the MRP1 family there is a third transmembrane domain and a large cytoplasmic loop joining it to the rest of the protein that are required for transport (23). In the case of P-glycoprotein, an exporter of a broad range of hydrophobic compounds, it is generally accepted that energy for transport is derived from ATP hydrolysis at each of the NBDs (24). The S₅Kₐ receptor chloride channel may be considered a member of the MRP1 family on the basis of sequence similarity (13), and the cystic fibrosis transmembrane conductance regulator chloride channel may be considered a member on the basis of homology of the NBDs (10). ATP hydrolysis by these two proteins has not been extensively characterized but photoaffinity labeling by 8-azido-ATP has been reported. Mutations in Walker motifs of either NBD strongly inhibit photolabeling by both 8-azido-[α-³²P]ATP and 8-azido-[γ-³²P]ATP. A, mutations in Walker A and B motifs (underlined). Leucine residues replacing the lysines and aspartates are indicated in black boxes. B, expression of these MRP1 mutants. Lysates of stable BHK cell lines expressing each variant were subjected to Western blotting with monoclonal antibody against MRP1 as probe. C, photolabeling with 8-azido-[γ-³²P]ATP. Membranes from these cells were incubated and photolabeled as in the previous experiments. Lane 1, 10 μg of wild-type MRP1; lane 2, 20 μg of K684L; lane 3, 10 μg of K1333L; lane 4, 10 μg of D1454L/E1455L. D, photolabeling by 8-azido-[γ-³²P]ATP. Lane 1, 10 μg of wild-type MRP1; lane 2, 20 μg of K684L; lane 3, 10 μg of K1333L; lane 4, 10 μg of D1454L/E1455L. E, ATP-dependent LTC₄ uptake by membrane vesicles containing wild-type (closed diamonds) and mutant MRPs: NBD1 Walker A lysine mutant K684L (open circles), NBD2 Walker A lysine mutant K1333L (open square), NBD2 Walker B aspartate mutant D1454L/E1455L (closed circles). The lack of significant uptake by the wild-type in the presence of AMP (open diamonds) instead of ATP is also indicated.
tagenesis of Walker motif amino acids of NBD1 in SUR1 prevented trapping of the 8-azido-ADP hydrolysis product but the same changes in NBD2 did not (24). The influence of ADP on photolabeling, however, was prevented by mutating those residues in NBD2 (25). Hence the NBDs of SUR1 apparently are not functionally equivalent. Less information is available for cystic fibrosis transmembrane conductance regulator, which does bind (26) and hydrolyze ATP (27), but trapping of N3ATP after photolabeling with N3[α-32P]ATP has been reported to occur predominantly at NBD1 (26).

In this study we have analyzed the photolabeling of MRP1 with N3[γ-32P]ATP which detects binding of the intact molecule before hydrolysis (and also phosphorylation if it occurs) and with N3[α-32P]ADP, which measures trapping of N3[α-32P]ADP formed by hydrolysis as well as trapping of the resubstrate. Consistent with this, the amount of photolabeling on MRP1 was much greater using the α-[32P] than the γ-[32P]-labeled substrate. The α labeling was found to be dependent on the presence of a divalent cation as is ATP hydrolysis (11, 12) and augmented by vanadate, which would be expected to promote trapping of N3ADP as N3ADP-vanadate (14, 20). Unexpectedly, however, it was found that labeling with the γ-[32P]-labeled compound was similarly stimulated by vanadate. This of course could not reflect N3ADP trapping. A previous study of ATP hydrolysis by MRP1 (19) had pointed to the presence of separate binding sites for ATP and ADP on the protein. Therefore, we postulated that enhancement of intact N3ATP binding to the ATP site by vanadate might occur as a consequence of an allosteric response to the increased trapping of N3ADP at the ATP site. This interpretation was supported by the observation that additional low concentrations of ADP caused a large increase in labeling with γ-[32P]-labeled N3ATP.

To physically identify these separate but interactive binding sites, fragments generated by partial trypsin digestion after photolabeling were identified by site-specific monoclonal antibodies. The results obtained showed that intact N3ATP bound exclusively at NBD1, whereas trapping of the hydrolysis product, N3ADP, occurred primarily at NBD2. These experiments are of exactly the same type as those used to show that the trapping of [α-32P]ADP after hydrolysis of [α-32P]ATP occurs to the same extent at each NBD of P-glycoprotein (14). Therefore it is reasonable to conclude that the greater sequence dissimilarity in the NBDs of MRP1 than in P-glycoprotein is reflected by greater functional distinction also. Despite this, the function of one domain is coupled to that in the other. This can be concluded from two facts. First, inactivation of either domain by mutagen-
Allosteric Interactions between the Two NBDs of MRP1

thesis of Walker motif residues greatly reduces the primary role of both, i.e. ATP binding at NBD1 and ADP trapping at NBD2. Second, the latter has a strong positive allosteric effect on the former. The sketch in Fig. 6 is an attempt to visualize events involved in nucleotide binding and hydrolysis as they relate to the transport of an organic anion by MRP1. Clearly the model is derived from the original one for P-glycoprotein formulated by Senior and colleagues (15). The modifications and extensions are made to accommodate the characteristics of nucleotide binding to MRP1 reported, some of which are quite different from those to P-glycoprotein. Since the two proteins do belong to separate families of the ABC superfamily, it may not be surprising that variations on the basic mechanism of the fundamental ABC units are exhibited. It will be of interest to learn if there are similar or further variations as other similar and dissimilar ABC proteins are examined. In the case of MRP1 and the closely related MRP2 further details should be discerned from studies of these purified proteins (11, 12, 28).

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