ATP Binding to the First Nucleotide-binding Domain of Multidrug Resistance Protein MRP1 Increases Binding and Hydrolysis of ATP and Trapping of ADP at the Second Domain*

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Multidrug resistance protein (MRP1) utilizes two non-equivalent nucleotide-binding domains (NBDs) to bind and hydrolyze ATP. ATP hydrolysis by either one or both NBDs is essential to drive transport of solute. Mutations of either NBD1 or NBD2 reduce solute transport, but do not abolish it completely. How events at these two domains are coordinated during the transport cycle have not been fully elucidated. Earlier reports (Gao, M., Cui, H. R., Lo, D. W., Grant, C. E., Almquist, K. C., Cole, S. P., and Deeley, R. G. (2000) J. Biol. Chem. 275, 13098–13108; Hou, Y., Cui, L., Riordan, J. R., and Chang, X. (2000) J. Biol. Chem. 275, 20280–20287) indicate that intact ATP is observed bound at NBD1, whereas trapping of the ATP hydrolysis product, ADP, occurs predominantly at NBD2 and that trapping of ADP at NBD2 enhances ATP binding at NBD1 severalfold. This suggested transmission of a positive allosteric interaction from NBD2 to NBD1. To assess whether ATP binding at NBD1 can enhance the trapping of ADP at NBD2, photoaffinity labeling experiments with \([\alpha^{32}P]8\text{-N}_3\text{ADP}\) were performed and revealed that when presented with this compound labeling of MRP1 occurred at both NBDs. However, upon addition of ATP, this labeling was enhanced 4-fold mainly at NBD2. Furthermore, the nonhydrolyzable ATP analogue, 5′-adenylylimidodiphosphate (AMP-PNP), bound preferentially to NBD1, but upon addition of a low concentration of 8-N\(_3\)ATP, the binding at NBD2 increased severalfold. This suggested that the positive allosteric stimulation from NBD1 actually involves an increase in ATP binding at NBD2 and hydrolysis there leading to the trapping of ADP. Mutations of Walker A or B motifs in either NBD greatly reduced their ability to be labeled by \([\alpha^{32}P]8\text{-N}_3\text{ADP}\) as well as by either \([\alpha^{32}P]\)- or \([\gamma^{32}P]\)-8-N\(_3\)ATP (Hou et al. 2000, see above). These mutations also strongly diminished the enhancement by ATP of \([\alpha^{32}P]8\text{-N}_3\text{ADP}\) labeling and the transport activity of the protein. Taken together, these results demonstrate directly that events at NBD1 positively influence those at NBD2. The interactions between the two asymmetric NBDs of MRP1 protein may enhance the catalytic efficiency of the MRP1 protein and hence of its ATP-dependent transport of conjugated anions out of cells.

Received for publication, July 27, 2001, and in revised form, November 30, 2001
Published, JBC Papers in Press, December 7, 2001, DOI 10.1074/jbc.M107133200

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* This work was supported by Grant CA98978 from NCI, National Institutes of Health. 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.

† The abbreviations used are: P-gp, P-glycoprotein; MRP1, multidrug resistance protein; NBD, nucleotide-binding domain; TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone; 8-N\(_3\)ADP, 8-azidoadenosine 5′-diphosphate; 8-N\(_3\)ATP, 8-azidoadenosine 5′-triphosphate; AMP-PNP, 5′-adenylylimidodiphosphate; NTP, nucleoside triphosphate; NDP, nucleoside diphosphate.
Allosteric Coupling of MRP1 Nucleotide-Binding Domains

hydrolysis product, ADP, occurring primarily at NBD2 (1, 2, 30). However, experiments to date have not clearly determined whether ATP bound at NBD1 is hydrolyzed and the product, ADP, trapped there in the presence of vanadate. To assess the interaction of the NDP directly we examined photolabeling with [α-32P]8-N3ADP. We have found now that [α-32P]-8-N3ADP labels NBD2 to a much greater extent than NBD1. This labeling by the nucleoside diphosphate was enhanced 4-fold by a low concentration of ATP. Hence it appears that binding of ATP at NBD1 promotes the association of ADP with NBD2. Furthermore, photolabeling by the non-hydrolyzable [α-32P]-8-N3AMP-PNP, which in the absence of other nucleotides occurred predominately at NBD1, was enhanced at NBD2 by a low concentration of 8-N3ATP. Thus NTP interaction with NBD1 consistently increased binding of NTP or NDP at NBD2, reflecting a positive allosteric action of NBD1 on NBD2.

EXPERIMENTAL PROCEDURES

Materials—Anti-mouse Ig conjugated with horseradish peroxidase was purchased from Amersham Biosciences Inc. Chemiluminescent substrate for Western blotting was from Pierce. Sodium orthovanadate, EDTA, EGTA, and pH 7.5 were purchased from Sigma. [32P]N3ATP, [32P]N3ADP, [α-32P]8-N3ATP, [α-32P]-8-N3AMP-PNP, and 8-N3ATP were purchased from Affinity Labeling Technologies. The Stratalinker UV Cross-linker 2400 model (wavelength, 254 nm) was from Stratagene.

Cell Culture and Stable Transfection of MRP1 in Baby Hamster Kidney Cells—Baby hamster kidney (BHK-21) cells were cultured at 37 °C in 5% CO2. Stable cell lines expressing wild-type and mutant MRP1s, R684A, D792A, K1333L, and D1454L/E1455L were established according to manufacturer recommendations.

Membrane Vesicle Preparations—MRP1-containing membrane vesicles were prepared according to the procedure described previously (2). Briefly, the cells collected by centrifugation were resuspended in membrane vesicle preparation buffer containing 10 mM Tris-HCl, pH 7.5, 250 mM sucrose, 0.2 mM MgC12, and 1 X protease inhibitors (2.5 μg/ml aprotinin, 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 pounds/square inch in a Parr N2 cavitation bomb. After releasing the pressure, the cell homogenate was adjusted to 1 mM EDTA. The homogenate was diluted 5-fold with 10 mM Tris-HCl and 25 mM sucrose, pH 7.5, and centrifuged at 1000 × g to remove nuclei and unbroken cells. The supernatant was overlaid on a 35% sucrose solution containing 10 mM Tris-HCl, pH 7.5, 2 mM ouabain, and 0.1 mM EGTA for 10 min at 37 °C. The amount of membrane vesicle, divalent cation, 32P-labeled nucleotide, and vanadate are resuspended in 20 mM Tris-HCl, pH 7.4, 1 mM EDTA and the amount of TPCCK-treated trypsin indicated in the figure legend. TPCCK-treated trypsin was prepared and diluted in 10 mM HCl. The same amount of 10 mM HCl was added in the control sample. The labeled membrane proteins were digested with TPCK-treated trypsin at 37 °C for 15 min, brought back to ice, dissolved in gel sample buffer, and electrophoresed.

RESULTS

Photolabeling of MRP1 with [α-32P]-8-N3ADP—We first determined if the vanadate trapping of [α-32P]-8-N3ADP by MRP1 that occurs after hydrolysis of [α-32P]-8-N3ATP (1, 2) can also be achieved by exposing membranes containing the protein to the labeled and derivatized nucleoside diphosphate directly. Fig. 1, A and B, indicate that this does occur and is absolutely dependent on the presence of a divalent cation just as in the case of ATP hydrolysis by this protein and trapping with vanadate of the transition state for hydrolysis (2). In all three cases Mn2+ is more potent than Mg2+ which supports labeling to a similar extent as Ni2+ but much more effectively than Ca2+. Of the labeling that occurs, approximately half is dependent on the presence of vanadate (Fig. 1, C and D) and presumably reflects the transition state complex as when [α-32P]-8-N3ATP is used. The vanadate independent half is a measure of the binding and retention of the nucleoside diphosphate. Sauna et al. (33) found that formation of the vanadate-trapped transition state intermediate of P-glycoprotein had a high energy of activation. Examination of the temperature dependence of its formation in MRP1 (Fig. 1, E and F) indicated this is also the case with this protein. While there is only a modest increase in vanadate-independent binding as temperature is increased, there is a steep increase in vanadate-dependent trapping, especially between 20 and 37 °C.

Earlier studies had shown that trapping of [α-32P]-8-N3ADP formed by MRP1 catalyzed hydrolysis of [α-32P]-8-N3ATP was more prevalent at NBD2 than at NBD1 (1, 2, 30). To assess the distribution of the trapping directly from [α-32P]-8-N3ADP, trypsin digestion was used to separate fragments derived from the two NBDs (Fig. 2). The NBD2-derived fragments were labeled more strongly than those from NBD1. These experiments demonstrated that a stable vanadate-induced nucleotide complex of MRP1 can be formed using [α-32P]-8-N3ADP and divalent cation which after photolabeling results in greater covalent labeling of NBD2 than NBD1. This is consistent with the finding that vanadate trapping of [α-32P]-8-N3ADP from hydrolysis of [α-32P]-8-N3ATP also occurred predominantly at NBD2 (1, 2, 30).

ATP Enhances Labeling of NBD2 with [α-32P]-8-N3ADP—Because we had found previously that the vanadate trapping of N3ADP which occurred at NBD2 allosterically promoted the binding of N3ATP at NBD1 (2), we wondered if the interaction between the two domains which this implies might also be detectable in the opposite direction. Therefore we tested the influence of both ATP and N3ATP on the trapping of [α-32P]-8-N3ADP (Fig. 3). Clearly biphasic responses were observed in both cases (Fig. 3, A–D). At low concentrations, ATP had a strong stimulatory effect; at 10 μM ATP, for example, there was an approximately 4-fold elevation in the labeling of the whole MRP1 protein. At higher concentrations this effect was overcome and labeling was inhibited probably in a competitive manner. Although its stimulatory influence was slightly less marked, ATP-N3ATP also enhanced the labeling, then inhibited as its concentration was increased. These relationships were remarkably similar to those seen when the influence of ADP on ATP binding was examined (2). To confirm that the nucleoside triphosphates were promoting the labeling by [α-32P]-8-N3ADP at NBD2, limited trypsin digestion was carried out. This revealed that most of the increased labeling did occur at NBD2 (Fig. 3E). These observations support the notion there is indeed a positive allosteric interaction in the direction from NBD1, where nucleoside triphosphate binding is most
FIG. 1. MRP1 photolabeling with $[\alpha^{32}\text{P}]$-8-N$_3$ADP under various conditions. The photolabeling was carried out in 10 µl of solution containing 10 µM $[\alpha^{32}\text{P}]$-8-N$_3$ADP (1 µCi), 10 µg of MRP1-containing membrane vesicles, and the components indicated in the following experiments. A, divalent cation dependence of $[\alpha^{32}\text{P}]$-8-N$_3$ADP labeling. All the reactions contained 800 µM vanadate and were incubated at 37°C for 10 min. Divalent cations and 1 mM EDTA without divalent cation are indicated above the lanes. The 45-kDa protein labeled by $[\alpha^{32}\text{P}]$-8-N$_3$ADP was also present in the membrane vesicles prepared from the non-transfected parental baby hamster kidney cells and cannot be recognized by several different antibodies against MRP1 protein. B, relative amounts of $^{32}\text{P}$ radioactivity associated with the MRP1 bands in the presence of different divalent cations. The amounts of $^{32}\text{P}$ radioactivity associated with the bands were determined by electronic autoradiography (Packard Instant Imager). Amount of labeling in the presence of 10 mM MgCl$_2$ was considered as 100%. The results for the samples without divalent cation, with 1 mM EDTA, 10 mM CaCl$_2$, and 10 mM NiCl$_2$ are the average of two independent experiments. The result for 10 mM MnCl$_2$ is the average of four experiments. C, vanadate dependence of $[\alpha^{32}\text{P}]$-8-N$_3$ADP labeling. All the reactions contained 10 mM MgCl$_2$ and were incubated at 37°C for 10 min. Vanadate concentrations were as indicated above the lanes. D, relative amounts of $^{32}\text{P}$ radioactivity associated with the MRP1 bands in the presence of different amounts of vanadate. Amount of labeling in the presence of 800 µM vanadate was considered as 100%. The data shown are the average of two independent experiments. E, temperature effects in the presence or absence of vanadate. The experiments were carried out with 10 mM MgCl$_2$ at temperatures indicated above the lanes in the absence (−) or presence (+) of 800 µM vanadate. F, relative amounts of $^{32}\text{P}$ radioactivity associated with the MRP1 bands under the conditions described in E. Amount of labeling in the presence of 800 µM vanadate at 37°C was considered as 100%. The data shown are the average of two independent experiments. The MRP1 band is indicated by the arrowhead at 190 kDa.
prominent, to NBD2, the predominant site of nucleoside diphosphate trapping. Significantly, although most photolabeling detected with \([\alpha^{32}P]8\text{-N}_3\text{ADP}\) as substrate also reflects trapping of its hydrolysis product, \([\alpha^{32}P]8\text{-N}_3\text{ADP}\), its stimulation by \(N_2\text{ATP}\) is not detectable because of simple displacement or dilution of \([\alpha^{32}P]8\text{-N}_3\text{ATP}\) by the non-radioactive \(8\text{-N}_3\text{ATP}\) (Fig. 3, A and B).

**Walker Motif Mutations Diminish Inter-domain Effects**—To gain further evidence that the vanadate trapped complex formed directly from \([\alpha^{32}P]8\text{-N}_3\text{ADP}\) was the same as that from \([\alpha^{32}P]8\text{-N}_3\text{ATP}\) and assess which NBD was involved in the ATP stimulation of labeling the influence of mutagenesis of the Walker A lysines and Walker B aspartates was tested. As with \([\alpha^{32}P]8\text{-N}_3\text{ATP}\) labeling (2) all these mutants trapped much less \([\alpha^{32}P]8\text{-N}_3\text{ADP}\) than the wild-type (Fig. 4, A and B), those in NBD1 having a somewhat greater effect than those in NBD2. However, in no case was labeling completely eliminated and hence it was possible to examine how the stimulatory effect of ATP was influenced by these mutations. Essentially the stimulation was much reduced in the NBD1 mutants, K684L and D792A (Fig. 4, C and D), and in the NBD2 mutants, K1333L and D1454L/E1455L (Fig. 4, E and F), and the stimulation effects were shifted to higher ATP concentrations (Fig. 4, C–F). Trypsin digestion of either \([\alpha^{32}P]8\text{-N}_3\text{ATP}\) or \([\alpha^{32}P]8\text{-N}_3\text{ADP}\)-labeled K1333L and D1454L/E1455L proved that the mutated NBD2 fragment can still be labeled (data not shown). These results presumably imply that the mutations of Walker motifs greatly diminish nucleotide binding abilities rather than abolish their binding completely.

**Influence of AMP-PNP on \([\alpha^{32}P]8\text{-N}_3\text{ADP}\) Labeling**—Since most observations indicated that just the binding of ATP, as distinct from binding, hydrolysis, and product trapping at NBD2, occurred at NBD1, we asked whether the non-hydrolyzable ATP analogue, AMP-PNP, would exert a similar stimulatory influence on \([\alpha^{32}P]8\text{-N}_3\text{ADP}\) labeling. Fig. 5, A and B, indicate that there is a much weaker stimulation than that caused by ATP. This result could be due either to the fact that hydrolysis of ATP at NBD1 is required for its full stimulatory action or that AMP-PNP simply binds with much lower affinity than ATP. To test the latter possibility, we compared its ability to compete with labeling by \([\alpha^{32}P]8\text{-N}_3\text{ATP}\) with that of ATP. As seen in Fig. 5, E and F, compared with Fig. 5, C and D, AMP-PNP competes very much less effectively than ATP, indicating that indeed it does bind less well. As AMP-PNP, like ATP, does interact preferentially at NBD1 (see below), its weak stimulation of \([\alpha^{32}P]8\text{-N}_3\text{ADP}\) binding at NBD2 very likely reflects this diminished binding. However, these observations do not eliminate the possibility that the ability of ATP to be hydrolyzed may contribute to its greater influence than the non-hydrolyzable AMP-PNP.

**\([\alpha^{32}P]8\text{-N}_3\text{AMP-PNP}\) Labeling of MRP1**—To directly characterize the interaction of AMP-PNP with MRP1, photolabeling with the radiolabeled 8-azido derivative was performed. Fig. 6A shows that this labeling is not very much influenced by vanadate as would be expected, as no hydrolysis product is formed to be trapped by the phosphate analogue. Hence this labeling reflects binding of the intact compound rather than formation of a complex approximating the transition state for hydrolysis. To attempt to confirm that its preferred site of binding was NBD1 as we suggested above, limited trypsin digestion was performed after labeling. Fig. 6C (lanes 1–4) indicates that in the absence of additional nucleotides this is the case. Interestingly, however, when 5 \(\mu\)M \(N_2\text{ATP}\) is also present, \([\alpha^{32}P]8\text{-N}_3\text{AMP-PNP}\) binding occurs predominantly at NBD2 (Fig. 6C, lanes 5–8). Effective competition of the binding of \(N_2\text{ATP}\) is not unexpected on the basis of the relative affinities of ATP and AMP-PNP revealed in Fig. 5 but the switch of \([\alpha^{32}P]8\text{-N}_3\text{AMP-PNP}\) binding from NBD1 to NBD2 was not anticipated. However, its interaction there was also found to be stimulated by \(N_2\text{ATP}\) (Fig. 7) much like the binding of \([\alpha^{32}P]8\text{-N}_3\text{ADP}\) at
NBD2. This may mean that when the favored substrate, N3ATP, is bound at NBD1, there is a conformational change at NBD2 resulting in increased \[^{32}P\]8-N3AMP-PNP binding there. That the sites of \[^{32}P\]8-N3AMP-PNP binding and \[^{32}P\]8-N3ADP trapping at NBD2 are likely to be the same is further indicated by the influence of vanadate (compare Fig. 7,
A and B, with C and D). Where vanadate is present (Fig. 7, C and D) to support trapping of \(N_3\)ADP formed on hydrolysis of 8-\(N_3\)ATP there is a competition by \(N_3\)ADP for the binding of \([\alpha^{32}P]8-N_3\text{AMP-PNP}\) to NBD2 and hence the stimulatory effect of 8-\(N_3\)ATP is less than in the absence of vanadate (Fig. 7, A and B) where there is no trapping of \(N_3\)ADP at NBD2.
FIG. 5. Influence of AMP-PNP on labeling with $[\alpha^{32P}]8$-N$_3$ADP. The photolabeling experiments were carried out in a 10 µl of solution containing 10 µg of MRP1-containing membrane vesicles, 10 mM MgCl$_2$ and 800 µM vanadate. A, AMP-PNP has little effect on $[\alpha^{32P}]8$-N$_3$ADP labeling. The photolabeling was carried out in a solution containing $10^{7}$ M $[\alpha^{32P}]8$-N$_3$ADP (1 µCi) and the concentrations of AMP-PNP indicated. B, quantitation of the experiment in A considering the amount of labeling without AMP-PNP as 100%. The data shown are the average of two independent experiments. C, ATP competes strongly with $[\alpha^{32P}]8$-N$_3$ATP labeling. The photolabeling was carried out in a 10 µl of solution containing 20 µM $[\alpha^{32P}]8$-N$_3$ATP (1 µCi) and the concentrations of ATP indicated. D, quantitation of the experiment in C. The data shown are the average of three independent experiments. IC$_{50}$ for the inhibition is ~47 µM. E, AMP-PNP is a weak inhibitor of $[\alpha^{32P}]8$-N$_3$ATP labeling. The photolabeling was carried out in a 10 µl of solution containing 20 µM $[\alpha^{32P}]8$-N$_3$ATP (1 µCi) and the concentrations of AMP-PNP indicated. F, quantitation of the experiment in E. The data shown are the average of two independent experiments. IC$_{50}$ for the inhibition is ~480 µM. 190 indicates the labeled 190-kDa MRP1 protein.
DISCUSSION

In a previous study we had found that the trapping of N3ADP at NBD2 of MRP1 promoted the binding of the nucleoside triphosphate at NBD1 (2). In the present study attention has been focused on the influence of the interaction of both hydrolyzable (N3ATP) and non-hydrolyzable (N3AMP-PNP) nucleotide triphosphate with NBD1 on nucleotide binding to NBD2. Several different sets of photolabeling experiments employing [32P]-8-N3ADP or [32P]-8-N3AMP-PNP as well as [32P]-8-N3ATP pointed to a strong positive allosteric interaction between NBD1 and NBD2. Initially it was found that the protein could be photolabeled with [32P]-8-N3ADP in a divalent cation dependent, orthovanadate-stimulated manner (Fig. 1). Hence as was recently shown with P-gp (33) vanadate trapping of N3ADP presumably as a mimic of a MgATP-P, post-hydrolysis transition state complex can occur when MRP1 is presented with N3ADP as well as with N3ATP where hydrolysis must first take place. The labeling with [32P]-8-N3ADP was found to occur predominantly at NBD2 (Fig. 2) just as was the case when [32P]-N3ATP was employed as substrate (1, 2). Thus these results confirmed that NBD2 was the main site of NDP trapping by MRP1. However, there are several recent indications largely from structural studies that each catalytic site in an ABC protein may include residues from both NBD sequences (34, 35). Therefore it may not be possible to simply ascribe the hydrolysis reactions entirely to the sites whose photolabeling is detected. Nevertheless, both in experiments where the two domains (N-half and C-half) were co-expressed using a single dual-expression baculovirus vector (1) or when separated from the intact protein by trypsin digestion (2) there was greater trapping of [32P]-8-N3ADP formed by hydrolysis of [32P]-8-N3ATP at NBD2 than NBD1, whereas bound intact [γ32P]-8-N3ATP was detected predominantly at NBD1 (1, 2). Labeling with [32P]-8-N3ATP at NBD2 is 2–3-fold greater than at NBD1 (2). These findings could be interpreted as reflecting immediate hydrolysis at NBD2 to form ADP that is trapped there by vanadate but little hydrolysis at NBD1 so that primarily binding of intact ATP is detected there. Results of our present experiments with [32P]-8-N3ADP do show that the trapped ADP-Vi complex can be formed at NBD1 although to a much lesser extent than at NBD2 (Figs. 2A and 3E).

It was then found that NDP trapping at NBD2 was strongly promoted by the addition of low concentrations of ATP or N3ATP (Fig. 3). This striking effect of itself and through the
subsequent experiments to which it lead shed a great deal of light on the allosteric interactions that occur between the two NBDs. It is important to emphasize that this effect was only detectable through the use of $[^a-32P]$8-N$_3$ADP or $[^a-32P]$8-N$_3$AMP-PNP as the labeling reagents even though the binding and hydrolysis of 8-N$_3$ATP at NBD2 is also promoted by the effects of these same low concentrations of ATP or 8-N$_3$ATP at NBD1 (see below). This stimulating influence, however, cannot be detected using $[^a-32P]$8-N$_3$ATP as labeling reagent since it is simply diluted by the addition of increasing concentrations of non-radioactive 8-N$_3$ATP (Fig. 3, F and G). This interpretation is supported by the finding that $[^a-32P]$8-N$_3$AMP-PNP labeling of NBD2 is also strongly stimulated by low concentrations of 8-N$_3$ATP (Figs. 6 and 7). In the absence of other added nucleotide 8-N$_3$AMP-PNP, like $[^a-32P]$8-N$_3$ATP (2), which preferentially labels NBD1 (Fig. 6C) and in so doing increases N$_3$ADP labeling of NBD2 (Fig. 6A). The labeling of NBD2 with $[^a-32P]$8-N$_3$ATP was not detected (2), simply indicating that the $[^a-32P]$phosphate in the bound $[^a-32P]$8-N$_3$ATP was removed by hydrolysis.

The non-hydrolyzable ATP analogue $[^a-32P]$8-N$_3$AMP-PNP was bound primarily to NBD1 in the absence of other nucleotides (Fig. 6C). This fact hints that NBD1 has higher affinity for nucleoside triphosphate than NBD2. Interestingly, however, binding to NBD2 was enhanced upon the addition of a low concentration of 8-N$_3$ATP (Fig. 6C). This change from greater binding at NBD1 to NBD2 probably reflected a higher affinity for 8-N$_3$ATP than AMP-PNP but the consequence for NBD2 is more interesting and appears to parallel the positive effect of ATP binding at NBD1 on N$_3$ADP labeling of NBD2 (Fig. 3E). Thus ATP or N$_3$ATP binding to NBD1 brings about enhanced binding of either hydrolyzable ATP or non-hydrolyzable AMP-PNP to NBD2. In the former case this results in an elevated amount of trapped ADP at NBD2 and in the latter case more bound AMP-PNP.

Since mutagenesis of the Walker A motif lysine and Walker B aspartate residues in the NBDs of MRP1 reduced but did not completely eliminate photolabeling by $[^a-32P]$8-N$_3$ADP, it was possible to evaluate their effect on the response of this labeling to the nucleoside triphosphate (Fig. 4). Essentially the stimu-
allosteric effect was greatly reduced and shifted to a requirement for higher concentrations of the NTP. These effects were somewhat expected since the residues in NBD1 were required as the site of action of the NTP and those in NBD2 for labeling by N\textsubscript{3}ADP.

In addition to providing stronger evidence for a positive allosteric interaction between NBD1 and NBD2 with respect to nucleoside triphosphate interaction, these experiments have further emphasized the differences between the properties of the two domains. While both are sites of ATP binding and hydrolysis, bound intact NTP is detected at NBD1 indicating that hydrolysis is less rapid there than at NBD2 where most NDP trapping is found when the protein is presented with either NTP or NDP. These distinctive properties of the two domains provide a fundamental asymmetry which may facilitate their allosteric coupling and thereby achieve increased overall efficiency of ATP hydrolysis and perhaps drug transport.

Acknowledgments—We thank Sharon Fleck for preparation of the manuscript and Marv Ruona for preparation of the graphics.

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