Conserved Walker A Ser Residues in the Catalytic Sites of P-glycoprotein Are Critical for Catalysis and Involved Primarily at the Transition State Step*

Received for publication, May 9, 2000, and in revised form, May 24, 2000
Published, JBC Papers in Press, May 30, 2000, DOI 10.1074/jbc.M003962200

Ina L. Urbatsch, Khursheed Gimi, Susan Wilke-Mounts, and Alan E. Senior‡

From the Department of Biochemistry and Biophysics, University of Rochester Medical Center, Rochester, New York 14642

P-glycoprotein (Pgp, also known as multidrug resistance protein) is a mammalian, plasma membrane-located protein of around 1280 amino acid residues, which has the ability to exclude and extrude a wide range of hydrophobic compounds from cells using the energy of ATP hydrolysis. It has particular relevance to use of chemotherapeutic drugs in cancer, and of protease inhibitor drugs for AIDS therapy, because it is able to prevent accumulation of many of these drugs in cells, thus conferring a multidrug-resistant phenotype (1–5). Consequently, there is currently a great deal of interest in development of clinically applicable methods to disable or circumvent Pgp in conjunction with drug therapies.

P-glycoprotein mutants S430A/T and S1073A/T, affecting conserved Walker A Ser residues, were characterized to elucidate molecular roles of the Ser and functioning of the two P-glycoprotein catalytic sites. Results showed the Ser-OH is critical for MgATPase activity and formation of the normal transition state, although not for initial MgATP binding. Mutation to Ala in either catalytic site abolished MgATPase and transition state formation in both sites, whereas Thr mutants had similar MgATPase to wild-type. Trapping of 1 mol of MgADP/mol of P-glycoprotein by vanadate, shown here with pure protein, yielded full inhibition of ATPase. Thus, congruent with previous work, both sites must be intact and must interact for catalysis. Equivalent mutations (Ala or Thr) in the two catalytic sites had identical effects on a wide range of activities, emphasizing that the two catalytic sites function symmetrically. The role of the Ser-OH is to coordinate Mg2+ in MgATP, but only at the stage of the transition state are its effects tangible. Initial substrate binding is apparently to an "open" catalytic site conformation, where the Ser-OH is dispensable. This changes to a "closed" conformation required to attain the transition state, in which the Ser-OH is a critical ligand. Formation of the latter conformation requires both sites; both sites may provide direct ligands to the transition state.

Pgp is a member of the ABC transporter superfamily (6). It contains two transmembrane domains, each consisting of six transmembrane helices (7, 8) and two ATP-binding sites (9–11) arranged in the linear sequence [TMD1]-[ATP1]-[TMD2]-[ATP2]. The binding sites for drugs and other hydrophobic compounds are located within the transmembrane domains (12–16), and both transmembrane domains appear to be required to provide correct drug-binding (11). Pgp shows ATPase activity, which is significantly stimulated by drugs, and reaches maximally a turnover rate of \(~10^{-1}\) s\(^{-1}\) (17–19). Experiments with vanadate (Vi) demonstrated that both of the ATP binding sites are capable of cleaving ATP and that the two sites interact in a highly cooperative mechanism of ATP hydrolysis (20, 21); this conclusion was supported by further work with the inhibitor beryllium fluoride (BeFx, Ref. 22). Later, it was shown that disruption of just one of the two sites by covalent chemical modification (23) or mutagenesis (24, 25) was sufficient to prevent even a single turnover of ATP hydrolysis at the other, intact site. These experiments supported a working model of the catalytic mechanism in which the two sites alternate to hydrolyze ATP (26). Further work using vanadate as a photocleavage agent (27) supported the model. Additionally, experiments in which drug binding was assessed using photoaffinity labeling by drug analogs (28–30) has supported the idea, introduced in Ref. 26, that changes in affinity at the drug binding site(s) are linked to formation and collapse of the catalytic transition state.

A detailed understanding, in molecular terms, of the structure of Pgp and the mechanism by which it hydrolyzes ATP and couples this process to drug transport, will lead to advances in overcoming multidrug resistance. No high resolution structure of Pgp is yet available, although in recent work we have described a method for large scale purification of detergent-soluble Pgp (31) that provides sufficient material for crystallization trials. Until high resolution data on Pgp are available, it seems reasonable to utilize the HisP x-ray structure (32) as a guide. HisP is the catalytic, ATP-hydrolyzing subunit of the bacterial ABC transporter, histidine permease (33). It shows significant sequence homology to the two Pgp ATP-binding sites, containing Walker A, Walker B, and ABC signature ("LSGGQ") sequences. In addition, HisP contains a Tyr residue (Tyr-16) which, in the x-ray structure, is stacked against the adenine ring of bound ATP. Tyr-16 corresponds in sequence to each of the two Tyr residues (Tyr-397 and Tyr-1040) that are covalently labeled by the photoaffinity label S-azido-ADP trapped in the N- and C-terminal ATP-binding sites of Pgp (34). Therefore, significant similarities in structure between the HisP and Pgp catalytic sites are evident.

* This work was supported by National Institutes of Health Grant GM50156 (to A. E. S.). 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.
‡ To whom correspondence should be addressed: Dept. of Biochemistry and Biophysics, Box 712, University of Rochester Medical Center, Rochester, NY 14642. Tel.: 716-275-2777; Fax: 716-271-2683; E-mail: alan_senior@urmc.rochester.edu.

The abbreviations used are: Pgp, P-glycoprotein; DM, n-dodecyl-β-D-maltoside; Vi, orthovanadate; AlF\(_3\), fluoroauremate; BeFx, beryllium fluoride complex; DTT, dithiothreitol; AMPPNP, adenosine 5’-(β,γ-imino)-triphosphate.
The Walker A consensus sequence (GXXXXGK(S/T)), found in numerous ATP- and GTP-hydrolyzing enzymes, ends in a highly conserved Ser or Thr. In cases where x-ray structures are available (e.g., myosin, ATP synthase F$_{\text{S}}$ sector, G-proteins), the hydroxyl oxygen of this residue is seen to provide a direct ligand to the Mg$^{2+}$ of the substrate MgNTP (35–37). Mg$^{2+}$ is not present in the HisP x-ray structure, because uncomplexed ATP was the ligand bound in the crystals, but Hwang et al. (32) argue that water 407 occupies the position that would normally be occupied by Mg$^{2+}$. They point out that water 407 lies 3.26 Å from one of the γ-phosphate oxygens of ATP, 3.37 Å from a β-phosphate oxygen, and 2.73 and 3.31 Å, respectively, from the two carboxyl oxygens of Asp-178, the Walker B Asp residue. Examination of the HisP structure shows that the hydroxyl oxygen of Ser-46 lies 2.90 Å from Water-407, and is therefore another candidate to provide a coordinating ligand, direct or indirect, to the Mg$^{2+}$ in the natural MgATP substrate. Ser-46 lies at the end of the HisP Walker A consensus sequence, and corresponds to Ser-430 and Ser-1073 in the N- and C-terminal ATP binding sites of Pgp.

Mg$^{2+}$ is an obligate cofactor for ATP hydrolysis in Pgp (18). To investigate the possible role of Ser-430 and Ser-1073 in Pgp catalysis, and to further study the role of the two catalytic sites, we generated mutations at these positions in mouse MDR3 Pgp.3 Mutant proteins were expressed in Pichia pastoris and purified to homogeneity (31).

**EXPERIMENTAL PROCEDURES**

**Introduction of S430A, S430T, S1073A, and S1073T Mutations into Mouse mdr3 cDNA—**Site-directed mutagenesis used the commercially available Altered Sites II method from Promega. For mutations in the N-terminal ATP-binding site, an EcoRI-EcoRI fragment of mouse mdr3 cDNA (base pairs 13–2248) was excised from pDR16 (38) and ligated into pAlter-1 to create pAlt-mdr3N, which was the template for mutagenesis. To facilitate subsequent manipulations, this template also contained engineered NruI (position 1348) and SalI (position 900) restriction sites. The mutagenic oligonucleotide for the S430A mutation was: GCGTGGAAAACGACAGCACTGTCG, in which the underlined GCC (Ala) replaced AGC (Ser) and introduced a PshAI site. For mutations in the C-terminal ATP-binding site, an EcoRI-AgeI fragment (base pairs 2248–4007) of mouse mdr3 cDNA was excised from pVT-mdr3.5 (24), the AgeI site was blunt-ended with T4 DNA polymerase, and the fragment was ligated into pAlter-1, which had been cut with NruI and SalI and blunt-ended at the SalI site, to create pAlt-mdr3C5, which was the template for mutagenesis. To facilitate subsequent manipulations, this template also contained engineered NruI (position 2872), SalI (position 2631), SpeI (position 3067), SnaBI (position 3980), and AgeI (position 4007) restriction sites. The mutagenic oligonucleotide for S1073A mutation was: GCGTGCGGAAAACGACAGCACTGTCG, where the underlined bases replace Ser (AGC) by Ala (GCC) and introduce a new PshAI site. The mutagenic oligonucleotide for S1073T mutation was: CGTCGCGGAAAACGACAGCACTGTCG, where the underlined bases replace Ser (AGC) by Thr (ACG) and introduce a new PshAI site. The S430T mutation was obtained as follows. The template was an EcoRI-EcoRI fragment (base pairs 13–2248) from mdr3 cDNA ligated in M13mp18 (24), and the mutagenic antisense oligonucleotide was: GGCAGACTGTTGTTTTTCCGAGC, in which the mutagenic Y changes Ser (TCC) to Ala (GCC) or Thr (ACC). Only Thr mutants were obtained. Mutagenesis in this case was by the T7-GEN method (U.S. Biochemical Corp.). In all cases, presence of the correct mutation and lack of any undesired mutations in the fragment to be transferred into pHIL-mdr3.5-His$_6$ were checked by DNA sequencing.

**Expression of Mutations in P. pastoris—**Expression of mutations utilized the Invitrogen P. pastoris expression system (license no. 272749). Mutations were transferred to plasmid pHIL-mdr3.5-His$_6$ by using the following restriction fragments: S430A and S430T, BgIII-SmaI; S1073A and S1073T, SpeI-SnaBI. Successful transfer was verified by restriction digestion and/or DNA sequencing. The new pHIL-mdr3.5-His$_6$ derivatives were carefully checked using a combination of AflII, BgIII, SmaI, and SnaBI, to ensure that they gave frag-

3 Mouse MDR3 Pgp is 87% identical in amino acid sequence to human MDR1 Pgp.
Vi-induced Trapping of Nucleotide Using [α-32P]ATP—Vi trapping experiments were performed as in the previous paragraph, except that 100 μM [α-32P]ATP replaced ATP. Control experiments using 100 μM [α-32P]ATP showed that <0.02% of applied nucleotide eluted from centrifuge columns in the absence of protein, all other components being present. The amount of trapped radioactive nucleotide was proportional to the amount of Pgp used in the range 3.5 μg (25 pmol) to 20 μg (142 pmol). Stoichiometry of Vi-trapped nucleotide in Pgp was calculated using a molecular mass of 141,000 Da.

Direct Photoaffinity Labeling with [α-32P]ATP—Activated Pgp (1 μg) was incubated in 2.5 or 100 μM [α-32P]ATP, 150 μM verapamil, 50 mM Tris-Cl, pH 7.5, in the presence of EDTA (1 mM) or MgCl2 as indicated, for 5 min on ice. Final DTT concentration was 1 mM. Samples were irradiated in a methacrylate cuvette for 3 min at room temperature with a xenon arc lamp (Osram XBO 450). 20 μl of irradiated sample (5 μg) was mixed with 10 μl of SDS buffer and run on 10% SDS gels. Gels were stained with Coomassie Blue and subjected to autoradiography. To quantify amount of radioactivity in the Pgp bands, a PhosphorImager (Storm 860, Molecular Dynamics) and software (ImageQuant 5.0, Molecular Dynamics) were used.

Photoaffinity Labeling with 8-Azido-[α-32P]ATP—Activated Pgp (5 μg) was incubated in 20 μl containing 2.5 μM 8-azido-[α-32P]ATP, 150 μM verapamil, 50 mM Tris-Cl, pH 7.5, in a total volume of 50 μl at 37 °C for 20 min. EDTA, MgCl2, MnCl2, CaCl2, or CoCl2 were included at concentrations indicated under "Results." Incubations were started by addition of 8-azido-[α-32P]ATP and stopped by transfer to ice. 50 μl of ice-cold 50 mM Tris-Cl, pH 7.5, was added to bring the volume to 100 μl, and free label was promptly removed by passage through a centrifuge column (above). Samples were irradiated on ice with UV for 5 min as above. 10 μl of SDS buffer was added, and samples were incubated for 10 min at 37 °C, then concentrated to 15-μl volume in a SpeedVac at 43 °C and run on SDS gels.

Routine Procedures—Protein concentrations were determined by the bichinchoninic acid method in presence of 1% SDS using bovine serum albumin as a standard (18). SDS-polyacrylamide gel electrophoresis was done using the Mini-PROTEAN II gel and Electrophoresis system (Bio-Rad). Samples (2 volumes) were dissolved in 1 volume of 5% (w/v) SDS, 25% (v/v) glycerol, 0.125 M Tris-Cl, pH 6.8, 0.01% pyronin Y, 160 mM DTT for 20 min at 37 °C, then run on 10% polyacrylamide gels. For immunodetection of Pgp, mouse monoclonal antibody C219 (Signet Laboratories Inc.) was used with the ECL detection system (Amersham Pharmacia Biotech). For autoradiography, SDS gels were stained with Coomassie Blue, dried, and exposed overnight at -80 °C to Kodak Biomax films with intensifying screens.

Materials—8-Azido-[α-32P]ATP was purchased from Affinity Labs Inc. [α-32P]ATP was from NEN Life Science Products. Acetone/ether-precipitated E. coli lipids were from Avanti Polar Lipids. Other chemicals were as described in Ref. 31.

RESULTS

General—Analysis of the catalytic mechanism of P-glycoprotein in molecular detail has been facilitated by two recent advances. One is the resolved x-ray structure of the nucleotide-binding subunit HisP from the ABC transporter histidine permease (32), which, as we argue in the Introduction, likely provides a reasonable facsimile of the structure of the nucleotide-binding domains of Pgp. The second is the development of a large panel of mutants for purification and study of mutant Pgp in detergent-soluble form (31). In this paper we study the role of conserved Ser residues (Ser-430 and Ser-1073), which we have identified by a large scale method for purification of mutant Pgp in detergent-soluble form (31). Modifications to the procedure were made to improve yield and speed, as noted under "Experimental Procedures." We routinely obtained a yield of -25 g of cells/liter of culture, and a final average yield of 0.03 mg of Pgp/g wet weight of cells for the four mutant proteins, as compared with 0.05 mg of Pgp/g wet weight of cells for wild-type Pgp grown under the same conditions. In a typical purification, we grew 3-liter batches of cells, and obtained 2 mg of pure mutant protein. Each mutant protein was purified at least twice, and excellent agreement was seen between replicate preparations in terms of specific ATPase activity. Fig. 1 shows an SDS gel of the purified mutant proteins. The results confirmed those obtained in Ref. 31, showing that this procedure provides extremely pure, detergent-soluble Pgp.

Characterization of MgATPase Activity of Mutant Proteins—Pgp was maximally activated by lipid and DTT before assay (see "Experimental Procedures"). Table I shows specific ATPase activities for wild-type and the four mutant proteins, in absence and presence of verapamil, along with K_m(MgATP), and K_app values for verapamil stimulation. Three conclusions are apparent. The first is that removal of the Ser hydroxyl by mutagenesis to Ala, in either ATP-binding site, reduces MgATPase activity to a negligible value. Therefore, there can be no doubt that the Ser hydroxyl plays a critical role in catalysis. The second is that mutagenesis to Thr has little impairing effect on specific MgATPase activity or K_m(MgATP), on degree of verapamil-stimulation of MgATPase, or on K_app values for verapamil. Therefore, the Thr hydroxyl is able to substitute for the Ser. Third, the same effects were seen for equivalent mutations in either ATP-binding site, confirming that two intact nucleotide sites are required for ATPase activity.

Metal Dependence of ATP Hydrolysis in Mutant Proteins—We next analyzed metal dependence of ATPase activity. In these experiments, NaATP concentration was fixed at 10 mM while metal concentration was varied. Metals tested were Mg2+, Mn2+, Co2+, and Ca2+. Fig. 2 shows results obtained for wild-type, S430T, and S1073T mutants. It is seen that maximal MgATPase activity of S430T and S1073T was around 80% of wild-type, and was obtained at equimolar concentration of Mg2+ ATP as in wild-type. With Mn2+ maximal activity occurred at 3–7.5 mM metal, and the Thr mutants had 1.1–1.2-fold higher activity than wild-type. With Ca2+ maximal activity occurred at equimolar concentration of ATP and metal, with the Thr mutants showing 1.8–2.0-fold higher activity than wild-type; with Co2+ maximal hydrolysis occurred at 0.2–0.3 mM metal, and the Thr mutants had 1.4–1.8-fold higher activ-
ity than wild-type. It should be noted that activity with Ca\(^{2+}\) and Co\(^{2+}\) was much lower than with Mg\(^{2+}\). With all metals, activity was stimulated by verapamil to the same extent in wild-type and mutant proteins (data not shown). The following conclusions are evident. First, wild-type mouse Pgp shows ATPase activity with all four metals, albeit with different apparent affinity and turnover rates. Second, the Thr mutants show parallel apparent affinities to wild-type for the four metals, but show different maximal turnover rates from wild-type, being higher in all cases except for the natural MgATP. Thus, interaction with divalent metals is different in the Thr mutants as compared with the wild-type Ser. Third, the two Thr mutants behaved very similarly, suggesting “symmetrical” behavior of the two nucleotide sites.

Fig. 3 shows results obtained with the two Ala mutants. Again, it was notable how mutation in N- or C-terminal sites gave the same effects. No significant ATPase activity was seen at any concentration of Mg\(^{2+}\) with either S430A or S1073A. Significant, albeit very low activity, was seen with Mn\(^{2+}\), at equimolar or higher concentration ratio of metal to ATP. This activity was not stimulated by verapamil (data not shown). The measured activity might be due to contaminant protein, however both mutant proteins showed Vi trapping of nucleotide by the Pgp band with different metals (see below). The main conclusion is that the Ser hydroxyl is critical for liganding and correct stereochemical orientation of Mg\(^{2+}\) to achieve normal turnover rates; however, Mn\(^{2+}\), Ca\(^{2+}\), and Co\(^{2+}\) at high concentration can support low level ATPase activity independently of the presence of the Ser hydroxyl.

### Table I

| Mutant Pgp | Specific ATPase activity | K<sub>n</sub>/MgATP<sup>a</sup> | K<sub>n</sub>/verapamil<sup>b</sup> |
|------------|-------------------------|-----------------|-----------------|
|            | µmol/min/mg verapamil   | µM              | µM              |
| Wild-type  | 0.3                     | 4.0 (13-fold)   | 0.44            |
| S430A      | NS<sup>c</sup>          | 3.3 (13-fold)   | 5.3             |
| S430T      | NS<sup>c</sup>          | NS              | NS              |
| S1073A     | NS<sup>c</sup>          | 3.2 (18-fold)   | 0.47            |
| S1073T     | 0.18                    | 0.18            | 5.0             |

<sup>a</sup> Verapamil was included at 150 µM, shown to be sufficient for maximum stimulation of activity. Fold-stimulation is shown in parentheses.

<sup>b</sup> K<sub>n</sub>/verapamil<sup>a</sup> is the concentration of verapamil required for 50% stimulation of activity, determined from curves of ATPase activity at pH 8.5 plotted versus verapamil concentration from 0 to 300 µM verapamil.

<sup>c</sup> NS, significant ATPase activity above background was not seen.

### Inhibition of MgATPase Activity of S430T and S1073T Mutant Pgp by Vanadate, Fluoroaluminate, and Beryllium Fluoride—Vi and AlFx are transition state analogs that trap MgADP tenaciously in the catalytic sites of Pgp, while BeFx is a ground-state analog that traps MgADP and mimics bound MgATP. All three analogs yield potent inhibition of wild-type Pgp-ATPase activity (20–22, 41). To test whether the transition state and MgATP-bound ground states of the Thr mutants differed from the wild-type, MgATPase activity was assayed as a function of concentration of Vi, AlFx, or BeFx. Table II shows IC<sub>50</sub> concentrations obtained. After maximal inhibition, rates of reactivation of ATPase were also measured and are presented as half-times in Table II. In all cases, full reactivation to original activity followed a single exponential process; results for the wild-type mouse protein were similar to previous data with hamster Pgp (41). The data show that the MgATPase transition state and MgATP-bound ground-state in the Thr mutants are similar to those achieved by wild-type. Because the Ala mutants have no detectable activity, this assay could not be used in those cases.

### Direct MgATP Photolabeling of Wild-type and Mutant Proteins—Direct photolabeling by [α-32P]ATP was used to assay MgATP binding properties of mutants. The procedure involved preincubation of Pgp with 2.5 or 100 µM [α-32P]ATP in presence of varied amounts of Mg\(^{2+}\), irradiation, and SDS-gel electrophoresis of irradiated protein (see “Experimental Procedures”). Fig. 4 shows results for wild-type, S430A, and S430T with 2.5 µM nucleotide. The same relative pattern was seen with 100 µM nucleotide. Extensive cross-linking of Pgp molecules was evident from the slower mobility bands (this was also evident from Coomassie Blue-stained gels). Quantitation of radioactivity by PhosphorImager analysis indicated that 2.1% of the Pgp molecules became labeled under optimal conditions, and if 1 mM concentrations of ATP or AMPPNP were included, photolabeling was reduced by 80% and 90%, respectively. From Fig. 4 it is clear that, in mutants and wild-type, degree of labeling was the same in the S430A, S430T, and wild-type Pgp. Therefore, lack of ATPase activity in the Ala mutant is not due to lack of MgATP binding. Results obtained with S1073A and S1073T mutants were also the same as for wild-type (data not shown). Fig. 4 shows that photolabeling was modulated by Mg\(^{2+}\) concentration, but that Ala and Thr mutants resembled wild-type in this property. Data in Fig. 4 were obtained with lipid-activated Pgp; similar data were evident in experiments using detergent-soluble Pgp with no added lipid.

### Photolabeling with Mg-8-Azido-[α-32P]ATP—We had shown...
showed the same modulation of 8-azido-ATP binding by increasing Mg\(^{2+}\) concentration as wild-type. This indicated that at the stage of substrate binding, Ser hydroxyl coordination to Mg\(^{2+}\) is not mandatory. The same feature was apparent also in direct MgATP photolabeling experiments (see above).

**Vi Trapping of Mg-[α\(^{32}\)]P/ADP—**Development of the centrifuge column procedure for use with pure Pgp allowed Vi trapping procedures to be carried out with the natural substrate MgATP, and for stoichiometry of trapped nucleotide to be calculated for the first time with pure protein. Fig. 6 shows that wild-type, S430T, and S1073T mutants each trapped Mg[-32P]ADP to the extent of 0.90–0.96 mol/mol of Pgp. Since ATPase in the eluted protein was inhibited by around 90%, this corresponds to 1 mol of trapped nucleotide/mol of pure Pgp at full inhibition. These data confirm previous work using hamster plasma membrane-bound Pgp (20). No trapping of Mg[-32P]ADP occurred in S430A and S1073A mutants, showing that, although they could bind MgATP (see above), these mutants could not attain the catalytic transition state, in either their mutated or intact nucleotide site.

Negligible radioactivity (0.01 mol of [32P]/mol of Pgp) eluted with wild-type or mutant Pgp if Vi was omitted, showing that all radioactive MgATP that bound to Pgp catalytic sites in the preincubation was lost during centrifuge column elution. This is not surprising since the separation time on 1-ml centrifuge columns is ~5 s (40), and it may be calculated from $K_m$ (MgATP) = 0.5 mM (Table I) and $k_{cat}/K_m = 10^4$ M\(^{-1}\) s\(^{-1}\) (42) that the dissociation rate for MgATP is ~5 s\(^{-1}\) ($t_{1/2}$ = 0.14 s); hence, the centrifuge column procedure is not suitable for direct nucleotide binding studies of Pgp, even with pure protein.

An additional point is that when wild-type Pgp containing Vi-trapped Mg[α\(^{32}\)]P/ADP was subjected to the direct photolabeling procedure as in Fig. 4, there was zero labeling of Pgp. This showed that direct photolabeling is strongly disfavored in the transition state conformation.\(^4\)

**Vi Trapping of 8-Azido-[α\(^{32}\)]P/ADP with Different Metals—**It was shown above that S430T and S1073T mutants showed a different spectrum of ATPase activities from wild-type with varied metal cofactors and, interestingly, that S430A and S1073A mutants, which were inactive with MgATP as substrate, nevertheless showed very low hydrolysis activity with MnATP, CoATP, and CaATP (Fig. 3). We used Vi trapping with 8-azido[α\(^{32}\)]P/ATP to test whether this activity was referable to Pgp and not to contaminant protein. Fig. 7 displays results obtained. With wild-type, S430T, and S1073T, Vi trapping occurred with all four divalent metals, but not in presence of EDTA. In wild-type, MnATP and MgATP yielded similar degree of trapping; CoATP and CaATP gave significantly lower trapping, reflecting their slower hydrolysis rates. Both Thr mutants resembled wild-type, as expected from their behavior in ATPase assays (Fig. 2). With S430A and S1073A mutants, no trapping was seen with Mg\(^{2+}\), agreeing with ATPase assays (Fig. 3), but low levels of trapping were seen with Mn\(^{2+}\) and Co\(^{2+}\). Consistent with the rapid rate of dissociation of Vi-trapped Mn-nucleotide and the slow rate of dissociation of Co-nucleotide (20, 21, 41), trapping was lower with Mn\(^{2+}\) than Co\(^{2+}\). The autoradiograms identify the very low ATPase activity with Mn- and Co-nucleotide as due to S430A and S1073A Pgp. However, no trapped nucleotide was evident with Ca\(^{2+}\), possibly because the rate of dissociation of Vi-trapped Ca-8-azido-ADP is rapid compared with the hydrolysis rate.

\(^4\) In contrast, photolabeling did occur with Vi-trapped Mg-8-azido-[α\(^{32}\)]P/ADP (Fig. 7). Whereas natural ATP assumes anti-conformation, 8-azido-ATP assumes syn-conformation, likely accounting for the difference.
Fig. 4. Direct photolabeling of mutant and wild-type Pgp by \([\alpha-\text{P}]\)ATP. Direct photolabeling was achieved by incubation of activated Pgp with 2.5 µM [\(\alpha-\text{P}\)]ATP and 150 µM verapamil in presence of varied concentration of MgCl\(_2\) (1 mM EDTA was present in the zero Mg\(^{2+}\) lanes) as described under “Experimental Procedures.” After irradiation, protein was run on SDS gels and subjected to autoradiography. A, wild-type Pgp; B, S430A mutant; C, S430T mutant.

Fig. 5. Photolabeling of wild-type and mutant Pgp by Mg-8-azido-[\(\alpha-\text{P}\)]ATP. Panels A and C–F, Pgp (2 µg) was incubated with 2.5 µM 8-azido-[\(\alpha-\text{P}\)]ATP and 150 µM verapamil in presence of increasing concentration of MgCl\(_2\) (the zero Mg\(^{2+}\) lane had 1 mM EDTA), irradiated by UV light, and the protein run on SDS gels then subjected to autoradiography. A, wild-type; C, S430A; D, S430T; E, S1073A; F, S1073T. Panel B, wild-type Pgp was incubated with 8-azido-[\(\alpha-\text{P}\)]ATP at 100 µM Mg\(^{2+}\), with 10-fold excess of indicated competing nucleotide (except 100-fold ATP in far right lane).

**DISCUSSION**

**General**—In this work we examined the mutants S430A, S430T, S1073A, and S1073T, affecting the Ser residue at the end of the Walker A sequence of the N- and C-terminal ATP-binding sites of mouse MDR3 P-glycoprotein. The goal was to understand the molecular role of this highly conserved Ser residue, and to further elucidate the functioning of the two Pgp ATP-binding sites. Three general conclusions are apparent. First, the results leave no doubt that both Ser hydroxyls are critical for catalysis because the Ala mutants had essentially zero MgATPase activity, whereas the Thr mutants had substantial, verapamil-stimulated MgATPase activity, with \(K_m\) (MgATP) similar to wild-type. The Ala mutants showed no Vi trapping of MgADP, demonstrating that they could not form the normal catalytic transition state, consistent with lack of MgATPase activity. The Thr mutants showed essentially normal Vi trapping of MgADP, and similar-to-normal inhibition by the transition state analogs Vi and AlFx, and the ground state analog BeFx. Reactivation after these inhibitions was also similar to normal. Second, the vanadate trapping results with the Ala mutants showed that both Pgp nucleotide sites must be intact for even a single catalytic turnover of MgATPase to occur in either site, indicating a mandatory requirement for cooperativity between the two sites, and confirming data obtained previously by chemical modification and mutagenesis of other critical residues in Walker A and B sequences (23–25). Third, no differences were seen between effects of the equivalent mutation (Ala or Thr) in the N- or C-terminal nucleotide-binding sites on a wide range of different activities. Examples of the activities measured were MgATPase, MnATPase, CoATPase, and CaATPase; direct photolabeling with MgATP; photoaffinity labeling with 8-azido-ATP; vanadate trapping of MgATPase, and vanadate trapping of 8-azido-ADP in presence of Mg\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\), or Ca\(^{2+}\). Therefore, in this work, the two nucleotide sites behaved symmetrically. Other workers have suggested that the two sites might behave asymmetrically (43).

**Molecular Role of the Ser Hydroxyl**—In MgATP hydrolizing enzymes, the essential Mg\(^{2+}\) cofactor is usually observed in octahedral coordination in the catalytic site, with the Ser/Thr at the end of the Walker A sequence providing a direct O ligand (44). Thus, mutagenesis to remove the hydroxyl group often leads to substantially decreased affinity for substrate MgATP (44). Here, however, photolabeling experiments did not reveal differences between mutant and normal in regard to MgATP or Mg-8-azido-ATP binding. Direct assays of MgATP binding by the centrifuge column technique proved unworkable due to the relatively rapid dissociation rate of the nucleotide. Therefore, we had to depend on other experimental approaches to investigate the potential role of Ser-430 and Ser-1073. Several pieces of evidence point to the fact that the Ser hydroxyls are involved in ligating Mg\(^{2+}\) in the transition state. First, there was no Vi
trapping of MgADP or Mg-8-azido-ADP in the two Ala mutants, although each bound MgATP and Mg-8-azido-ATP as indicated by photolabeling experiments. Normal Vi trapping occurred in the two Thr mutants. Second, the Thr mutants did support ATPase activity, but use of different divalent metals Mg$^{2+}$, Mn$^{2+}$, Co$^{2+}$, and Ca$^{2+}$ showed that, depending on the metal, the hydrolysis rate was different from wild-type, in several cases higher, consistent with the idea that the hydroxyl is not in exactly the same position in the Thr mutants as in wild-type. Third, even in the Ala mutants, where there was no evidence of any activity with Mg$^{2+}$, high concentrations of Mn$^{2+}$, Co$^{2+}$, and Ca$^{2+}$ “forced” a low ATPase activity, that was shown to be referable to Pgp. Possibly, these larger metals can recruit additional ligand(s), which are unavailable to Mg$^{2+}$.

Concept of an “Open Conformation” Binding Site That Binds Substrate Changing to a “Closed Conformation” Binding Site That Hydrolyzes It—Nucleotide binding to Pgp catalytic sites occurs with relatively low affinity, as evidenced by the $K_m$(MgATP) of around 1 mM seen here and in many previous reports, and by direct measurement of $K_f$(MgATP) by fluorescence quench technique, which gave a value of 0.46 mM (45). As noted by several workers, this indicates that the sites are relatively “open” when binding nucleotide. The x-ray structure of HisP supported this notion, showing ATP bound to a surprisingly open binding surface, different from the sequestered type of pocket usually seen in nucleotide-binding proteins. Although it is true that the HisP structure lacks Mg$^{2+}$, another ABC transporter x-ray structure, of the C-terminal nucleotide-binding domain of RbsA containing bound MgADP, also shows a relatively open binding surface for the nucleotide.

It is remarkable how consistently mutations of highly conserved residues, which are expected by analogy with other ATPase enzymes to contact the substrate MgATP, fail to bring about significant decreases in substrate binding affinity in Pgp. In this work, mutagenesis of the Walker A Ser-430 and Ser-1073 side chains to Ala had no effect on relative labeling by substrates MgATP or Mg-8-azido-ATP. In previous work, mutagenesis of the Walker B residues Asp-551 and Asp-1196 to Asn had little effect on MgTNP-ATP or MgTNP-ADP binding (31) or on Mg-8-azido-ATP labeling (24). Further, mutagenesis of the Walker A Lys residues (Lys-429 and Lys-1072) to Arg or Met had little effect on binding of MgTNP-ATP or MgTNP-ADP (31), or on labeling by Mg-8-azido-ATP (24, 46, 47). The roles of these highly conserved side chains in other ATPase enzymes are well established, and are to coordinate the essential Mg$^{2+}$ cofactor, either directly or indirectly through a bound water (Walker A Ser and Walker B Asp), and to ligand the βγ phosphate moiety of the nucleoside triphosphate electrostatically and by hydrogen bonding (Walker A Lys). Lack of significant effect of mutations of these residues on substrate binding in Pgp suggests that they are “engaged” only loosely with the substrate during the initial binding step.

In sharp contrast, and consistent with results in other systems, mutagenesis studies reveal that each of these residues is absolutely critical for attainment of the Pgp catalytic transition state, as measured by ATP hydrolysis and Vi trapping of Mg-nucleoside diphosphate. As the hydrolysis reaction proceeds from substrate binding to the catalytic transition state, the Walker A Lys and Ser and the Walker B Asp side chains must closely approach and engage the substrate. Indeed, removal of any one of these side chains is sufficient to prevent the transition state from being achieved, indicating a highly cooperative mechanism for formation of the transition-state structure, requiring a sequestered, closed conformation. The trigger for conversion of the open binding conformation to the closed active conformation is likely the binding of substrate to both ATP sites together with binding of drug at the inner leaflet drug-binding site. The active conformation requires interaction of both sites.

It is interesting to speculate how the closed active conformation might be achieved. Only one nucleotide site appears to form the transition state at any one time, as shown by the fact that the stoichiometry of Vi-trapped nucleoside diphosphate is 1 mol/mol of Pgp (Refs. 20 and 21, and this work). One explanation could be that one site at a time undergoes a large convolution to sequester the nucleotide in an enclosed pocket within itself. A more intriguing idea, which perhaps better accounts for the requirement for two intact nucleotide sites for even a single ATPase turnover, is that the transition state requires contributing side chain ligands from both nucleotide domains, meaning that the two domains must come together to allow residues from one site to project into the other. Allostery activation of ArsA ATPase has been proposed to be due to a conformational change that forms a tight interfacial contact between the two nucleotide binding domains (48). We believe this concept is interesting and likely productive for further structural and biochemical investigations of Pgp.

Acknowledgment—We thank Maria Tulaveva for excellent technical assistance.

REFERENCES
1. Ambudkar, S. V., Dey, S., Hrycyna, C. A., Ramachandra, M., Pastan, I., and Gottesman, M. M. (1999) Annu. Rev. Pharmacol. Toxicol. 39, 361–398.
2. Sharam, F. J. (1977) J. Membr. Biol. 160, 161–175.
3. Stein, W. D. (1997) Physiol. Rev. 77, 545–590.
4. C. S. Stauffacher, personal communication.

5. It should be noted that, in Ref. 43, labeling of membrane-bound Pgp by Mg-8-azido-ATP was shown to be impaired in the D551N mutant, although the D1196N mutant was not impaired. Reasons for the difference in regard to the D551N mutant between this and previous work (24) are not yet clear.
