Covalent Modification of Human P-glycoprotein Mutants Containing a Single Cysteine in Either Nucleotide-binding Fold Abolishes Drug-stimulated ATPase Activity*

(Received for publication, May 24, 1995, and in revised form, August 1, 1995)

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The ATPase activity of P-glycoprotein is inactivated by N-ethylmaleimide (NEM), which is postulated to modify cysteine residues within either of the homology A consensus sequences for nucleotide binding (GNS-GCGKS and GSSGCQKS, respectively) (Al-Shawi, M. K., Urbatsch, I. L., and Senior, A. E. (1994) J. Biol. Chem. 269, 8986–8992). To test this postulate as well as to determine the contribution of either nucleotide-binding domain to function, a Cys-less mutant was constructed, and then a single cysteine residue was reintroduced back into each nucleotide-binding consensus sequence. We then tested the sensitivity of the ATPase activity of each mutant to covalent modification by NEM. It was found that covalent modification of a single cysteine residue within either nucleotide-binding consensus sequence (Cys-431 and Cys-1074, respectively) with NEM inhibited drug-stimulated ATPase activity of P-glycoprotein. The concentrations of NEM required for half-maximal inactivation of ATPase activity were 7 and 35 μM for mutants Cys-431 and Cys-1074, respectively. In both cases, inactivation of ATPase activity by NEM was prevented by ATP. These results suggest that both nucleotide-binding domains may need to bind ATP to couple drug binding to ATPase activity.

P-glycoprotein, also known as the multidrug-resistance protein, is a plasma membrane protein that is involved in the ATP-dependent efflux of a broad range of cytotoxic hydrophobic compounds from cells (reviewed by Endicott and Ling, 1989; Roninson, 1991; and Gottesman and Pastan, 1993). Many of the drugs used in cancer chemotherapy such as vinblastine, vincristine, taxol, doxorubicin, and daunomycin are substrates of P-glycoprotein. Therefore, this protein has significant clinical importance, since it may be one of several mechanisms responsible for the development of multidrug resistance to chemotherapeutic drugs during treatment of cancers.

In this study, we used inactivation of ATPase activity by NEM to address the following questions: 1) Are the cysteines within the homology A regions (Cys-431 and Cys-1074, respectively) modified by NEM? 2) Is a mutant lacking these two cysteines still sensitive to inactivation by NEM? 3) If these two cysteines do react with NEM, then what is the effect of modifying only one of these residues? and 4) Does modification of the two cysteines outside the homology A regions (Cys-1125 or Cys-1227) affect ATPase activity?

Our approach was to utilize a Cys-less mutant of P-glycoprotein, which we have previously shown to retain the ability to confer drug resistance in transfected cells (Loo and Clarke, 1995). We then reintroduced a cysteine residue at its original position in the nucleotide-binding domains and assayed for ATPase activity after incubation with NEM. We found that inactivation of ATPase activity was indeed due to covalent modification of cysteine residues within the homology A sequences and that modification of either cysteine residue was sufficient to inactivate drug-stimulated ATPase activity of P-

* This research was supported by a grant (to D. M. C.) as part of a group grant from the Medical Research Council of Canada. 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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1 The abbreviations used are: NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; biotin maleimide, 3-(N-maleimidopropionyl) biocytin.
glycoprotein. These results suggest that both nucleotide-binding domains are capable of interacting with ATP and that both domains are essential for drug-stimulated ATPase activity.

**EXPERIMENTAL PROCEDURES**

Oligonucleotide-directed Mutagenesis—A full-length MDR1 cDNA cloned from a human kidney cortex cDNA library (Bell et al., 1986) and modified to encode the epitope for monoclonal antibody A52 (Zubrzycka-Gaarn et al., 1984) at the COOH-terminal end of the protein was inserted into the mammalian expression vector pMT21, as previously described (Loo and Clarke, 1993a). The sequence at the COOH terminus of P-glycoprotein that would normally end as TKRQ now became TkräislnscspefddlplaeqarcrGdpr. Oligonucleotide-directed mutagenesis was carried out as previously described (Loo and Clarke, 1993a). The construction of a Cys-less mutant of P-glycoprotein-A52, in which the codons for cysteine residues were simultaneously mutated to alanine, was previously described (Loo and Clarke, 1995).

Expression in Sf9 Cells with a Baculovirus Vector—The cDNAs coding for wild-type and Cys mutants of P-glycoprotein-A52 were subcloned into the multiple cloning site of Autographa californica nuclear polyhedrosis virus transfer vector pBlueBacIII (Invitrogen), downstream from the polyhedrin promoter. Preparation of virus stocks and infection of Sf9 cells with recombinant baculovirus was carried out as previously described (Loo and Clarke, 1994b).

ATPase Activity—Membranes from Sf9 cells infected with recombinant or control baculovirus were prepared as previously described (Loo and Clarke, 1993a) and suspended in Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) at a protein concentration of 1–2 mg/ml. The ATPase activity in the membranes was determined by incubating the membranes (10 μg protein) in buffer containing 100 mM Tris-HCl, pH 7.5, 4 mM EGTA, 4 mM dithiothreitol, 100 mM KCl, 10 mM Na2HPO4, 10 mM MgCl2, 2 mM ouabain, 5 mM ATP (Sarkadi et al., 1992), and various amounts of verapamil at 37 °C for 30 min. The amount of inorganic phosphate liberated was determined by the method of Chifflet et al. (1980).

Labeling with Biotin Maleimide—Membranes from Sf9 cells infected with recombinant baculovirus were incubated with biotin maleimide (3-(N-maleimidomethyl)propionamido)biocytin, Molecular Probes Inc.) in Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl, pH 7.5). Biotin maleimide was added from a stock prepared in Me2SO. The concentration of Me2SO in the labeling medium did not exceed 1% (v/v). After 10 min at room temperature, 2-mercaptoethanol was added to a final concentration of 4% (v/v), and the mixture was incubated a further 10 min at room temperature. The samples were then diluted 10-fold with lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate). Insoluble material was removed by centrifugation at 15,000 × g for 15 min. The supernatant fractions were incubated with monoclonal antibody A52 (Loo and Clarke, 1995). The immunoprecipitates were subjected to SDS-PAGE and transferred onto nitrocellulose; biotinylated proteins were detected using streptavidin-conjugated horseradish peroxidase and chemiluminescence (Amersham).

**RESULTS**

Construction and Expression of Mutants—Wild-type human P-glycoprotein contains seven cysteines (Fig. 1); three are located in the transmembrane domains (Cys-137, Cys-717, and Cys-956), one is located in the NH2-terminal nucleotide-binding domain (Cys-431), and three are in the COOH-terminal nucleotide-binding domain (Cys-1074, Cys-1125, and Cys-1227). In a previous study, we created a Cys-less mutant of P-glycoprotein-A52 by simultaneously changing all of the cysteine residues in the nucleotide-binding domains (Fig. 1). In a previous study on the topology of P-glycoprotein, we found that Cys residues located in the predicted transmembrane regions (Cys-137, Cys-717, Cys-956) were not labeled with biotin maleimide. Therefore, inactivation of P-glycoprotein ATPase activity by NEM (Al-Shawi and Senior, 1993) must involve the covalent modification of one or more Cys residues in the nucleotide-binding domains of P-glycoprotein. To identify the cysteine residues that were sensitive to modification by NEM, it was necessary to measure the ATPase activity of the mutant P-glycoproteins. Measurement of ATPase activity requires a relatively large amount of P-glycoprotein, which is difficult to achieve in mammalian cells. It has, however, been demonstrated that expression of P-glycoprotein in insect cells yields relatively large amounts of the transporter, which exhibits high levels of drug-stimulated ATPase activity (Germann et al., 1990; Sarkadi et al., 1992; Loo and Clarke, 1994b; Rao, 1995). Accordingly, we subcloned the cDNA coding for each of these mutant P-glycoproteins into a baculovirus expression vector for expression in cultured Sf9 insect cells.

Recombinant baculoviruses containing the cDNA coding for the various Cys P-glycoprotein mutants were used to infect Sf9 insect cells. 5 days after infection, membranes were prepared from the infected cells. Fig. 2 shows an immunoblot of membranes prepared from the infected cells. An immunoreactive protein of apparent mass of 140 kDa was the major protein, and it was found to be present in similar amounts in cells expressing either wild-type P-glycoprotein-A52 or the various Cys mutants. This product has an apparent mass similar to that of the underglycosylated form of the mature P-glycoprotein and is consistently observed to be present when expressed in insect cells (Germann et al., 1990; Loo and Clarke, 1994b, Rao, 1995). In mammalian cells, P-glycoprotein is more extensively glycosylated and migrates on SDS-PAGE gels with an apparent mass of 170 kDa. The lack of cysteines did not appear to contribute significantly to instability of the protein since similar amounts of wild-type and mutant forms of the enzyme were expressed, with no significant difference in the amount of proteolytic degradation products.

Verapamil-stimulated ATPase Activity—It has been found that verapamil, vinblastine, and colchicine stimulate the ATPase activity of P-glycoprotein, with maximal stimulation occurring in the presence of verapamil (Sarkadi et al., 1992; Ambudkar et al., 1992; Homolya et al., 1993; Al-Shawi and Senior, 1993). Stimulation of ATPase activity by various substrates correlates in many cases, with their transport by P-glycoprotein (Homolya et al., 1993), suggesting that coupling of drug binding to ATPase activity is related to drug transport. Accordingly, the effect of various concentrations of verapamil

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2 T.W. Loo and D.M. Clarke, unpublished data.
on the ATPase activity of wild-type and Cys mutants of P-glycoprotein was examined. As shown in Fig. 3, the ATPase activities of wild-type and mutant forms of P-glycoprotein were stimulated over a wide range of verapamil concentrations (0.1–10 μM). Maximal stimulation occurred at about 10–25 μM verapamil in all cases. The $K_a$ (half-maximal ATPase activating concentration) for wild-type P-glycoprotein was approximately 4.5 ± 1.3 μM. Similar results were obtained with all of the Cys mutants (3.5–4.5 μM). Therefore, the absence of Cys residues in the Cys-less mutant did not markedly alter the characteristics of verapamil stimulation of ATPase activity. There was also no significant difference when vinblastine-stimulated ATPase activities were measured (data not shown).

Inactivation of ATPase Activity with N-Ethylmaleimide—The ATPase activity of P-glycoprotein can be inhibited by covalent modification by NEM, and it was postulated that NEM reacts with the cysteine residues within the homology A nucleotide-binding consensus sequences (Al-Shawi et al., 1994). Accordingly, we attempted to determine which of the cysteine residues in the nucleotide-binding domains could be covalently modified by NEM. Fig. 4A shows the effect of NEM on the verapamil-stimulated ATPase activities of the wild-type and Cys mutants of P-glycoprotein. A 50% reduction in ATPase activity of the wild-type enzyme was obtained at about 7 μM NEM, which is comparable to the value of 5 μM reported by Urbatsch et al. (1994) using purified P-glycoprotein from Chinese hamster ovary cells. The Cys-less mutant of P-glycoprotein, however, was resistant to NEM, with more than 80% of the ATPase activity still present even at an NEM concentration of 1 mM. This result indicates that NEM is specific for cysteine residues. A similar conclusion was made by Senior et al. (1995), based on the kinetics and pH dependence of NEM modification of P-glycoprotein from Chinese hamster cells. The ATPase activities of mutants with a single cysteine residue within either homology A region (Cys-431 and Cys-1074, respectively) were sensitive to inhibition by NEM. Inhibition of the ATPase activity of mutant Cys-1074 resembled that of wild-type enzyme, with a half-maximal inhibition occurring at about 35 μM NEM. Mutant Cys-1125 and Cys-1227 were not sensitive to modification by NEM as they retained at least 80% of their ATPase activity after incubation with 1 mM NEM. These results show that inhibition of the ATPase activity by NEM is the result of modification of either Cys-431 or Cys-1074. It appears, however, that Cys-431 is less accessible to modification by NEM than Cys-1074, since more than five times the concentration of NEM was required to inhibit the former by 50%.

It is possible that NEM inhibition of drug-stimulated ATPase activity could be different from that of NEM inhibition of the constitutive ATPase activity of P-glycoprotein. Accordingly, we tested the effect of NEM on the ATPase activity of wild-type P-glycoprotein and the Cys mutants in the absence of verapamil. Fig. 4B shows that the pattern of inhibition of ATPase activity by NEM is similar to that observed in the presence of verapamil (Fig. 4B).

Labeling with Biotin Maleimide—To determine that Cys-1074 was indeed more accessible to NEM, labeling experiments were carried out with biotin maleimide to directly monitor labeling of the protein. Membranes prepared from infected Sf9 cells and containing equivalent amounts of P-glycoprotein-A52 were incubated with various concentrations of biotin maleimide for 10 min. The reactions were stopped by addition of 2-mercaptoethanol. The labeled membranes were then solubilized with detergent and P-glycoprotein-A52 immunoprecipitated with monoclonal antibody A52. The immunoprecipitates were subjected to SDS-PAGE, and biotinylated P-glycoprotein-A52 was detected using streptavidin-conjugated horseradish peroxidase and enhanced chemiluminescence (Amersham). Fig. 5 shows that labeling of mutant Cys-1074 occurred at a much lower concentration (0.5 μM) and was more pronounced at a concentration of 1 μM biotin maleimide than for Cys-431. Biotin maleimide was specific for cysteines since no labeling of the Cys-less P-glycoprotein was detected even at a concentration of 10 μM.

The presence of MgATP has been shown to prevent inhibition of the ATPase activity of P-glycoprotein by NEM (Al-Shawi et al., 1994). Accordingly, we tested whether ATP could also prevent inhibition of the ATPase activity of the Cys mutants by NEM. Fig. 6 shows the results of the ATP protection assays. The activities of wild-type and mutants Cys-431 and Cys-1074 P-glycoprotein were all protected from inactivation by NEM in the presence of MgATP. In all three cases, maximal protection was achieved in the presence of 2 mM ATP.

The ATPase activities of mutants Cys-1125 and Cys-1227 were relatively insensitive to NEM (Fig. 4). These results suggest that labeling of either of these cysteine residues by NEM either did not interfere with function or that they may be
inaccessible to covalent modification. To distinguish between these two possibilities, membranes prepared from Sf9 cells expressing equivalent amounts of P-glycoprotein-A52 were labeled in the presence of 10 μM biotin maleimide. As shown in Fig. 7, mutants Cys-431 and Cys-1074 were again modified by biotin maleimide, while little or no labeling was observed with the Cys-less mutant or mutants Cys-1125 or Cys-1227. These results suggest that residues Cys-1125 and Cys-1227 are inaccessible to biotin maleimide and are likely to be hidden beneath the surface of the nucleotide-binding domain.

**DISCUSSION**

The results of this study show that inactivation of the ATPase activity of P-glycoprotein by NEM occurs through covalent modification of cysteine residues within the homology A regions of the nucleotide-binding domains. In this study, inactivation refers to the loss of ATPase activity. The relationship between ATPase activity and substrate transport has not yet been resolved. In some cases, there is a good correlation between the ability of a substrate to be transported by P-glycoprotein and its ability to stimulate ATPase activity. For example, Homoya et al. (1993) showed that transport of various fluorescent compounds by P-glycoprotein correlated with their ability to stimulate ATPase activity of P-glycoprotein. Recently, however, Sharom et al. (1995) reported that the level of stimulation of the ATPase activity of P-glycoprotein by hydrophobic peptides did not correlate with their affinity as transport substrates. In addition, it appears that the extent of ATPase stimulation by various drug substrates can be influenced by the host lipid environment (Doige et al., 1993; Urbatsch and Senior, 1995). Further studies will be needed to
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The finding that only the cysteines within the homology A regions of the nucleotide-binding domains are accessible to covalent modification makes them ideal targets for reporter molecules for studying conformational changes during transport. These studies are currently in progress.

Acknowledgment—We are grateful to Dr. David H. MacLennan for the A52 epitope and monoclonal antibody used in this study.

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J. Biol. Chem. 1995, 270:22957-22961.
doi: 10.1074/jbc.270.39.22957

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