Cysteines 431 and 1074 Are Responsible for Inhibitory Disulfide Cross-linking between the Two Nucleotide-binding Sites in Human P-glycoprotein*

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Human wild-type and Cys-less P-glycoproteins were expressed in Pichia pastoris and purified in high yield in detergent-soluble form. Both ran on SDS gels as a single 140-kDa band in the presence of reducing agent and showed strong verapamil-stimulated ATPase activity in the presence of added lipid. The wild type showed spontaneous formation of higher molecular mass species in the absence of reducing agent, and its ATPase was activated by dithiothreitol. Oxidation with Cu(II) generated the same higher molecular mass species, primarily at 200 and ~300 kDa, in high yield. Cross-linking was reversed by dithiothreitol and prevented by pretreatment with N-ethylmaleimide. Using proteins containing different combinations of naturally occurring Cys residues, it was demonstrated that an inhibitory intramolecular disulfide bond forms between Cys-431 and Cys-1074 (located in the Walker A sequences of nucleotide-binding sites 1 and 2, respectively), giving rise to the 200-kDa species. In addition, dimeric P-glycoprotein species (~300 kDa) form by intermolecular disulfide bonding between Cys-431 and Cys-1074. The ready formation of the intramolecular disulfide between Cys-431 and Cys-1074 establishes that the two nucleotide-binding sites of P-glycoprotein are structurally very close and capable of intimate functional interaction, consistent with available information on the catalytic mechanism. Formation of such a disulfide in vivo could, in principle, underlie a regulatory mechanism and might provide a means of intervention to inhibit P-glycoprotein.

P-glycoprotein (Pgp) is a plasma membrane-located member of the ABC transporter family, which hydrolyzes ATP and uses the energy to pump a wide variety of drugs and other hydrophobic compounds out of cells. It is a major contributor to multidrug resistance in mammalian cells and is recognized as an impediment to therapy with anticancer and anti-AIDS drugs (1–6). Pgp consists of ~1280 amino acid residues, with the domain structure TMD1-NBS1-TMD2-NBS2, where TMD indicates a membrane domain consisting of six predicted transmembrane helices, and NBS1 and NBS2 indicate N- and C-terminal nucleotide-binding sites of Pgp, respectively, containing Walker A, Walker B, and ABC transporter signature consensus sequences. The drug-binding sites are proposed to be formed from several transmembrane helices in the membrane domains (7–11). ATP hydrolysis in the two nucleotide sites was proposed to occur via an alternating sites mechanism in which formation and collapse of the catalytic transition state are coupled to movement of drug across the membrane from the inner to outer surface (12). Considerable support for this mechanism has come from studies using the transition state analog MgADP- vanadate (13–16) and the ground state analog MgADP-beryllium fluoride (17) and additionally from chemical modification (18, 19) and specific mutagenesis of the catalytic sites (19, 20). Understanding the structure and mechanism of action of Pgp in detail is fundamental to devising ways to circumvent multidrug resistance in human cells, and this is an important goal not only because of its application to cancer and AIDS therapies, but also through its relevance to discovery of efficacious new drugs in general.

ATP hydrolysis in hamster or human Pgp is strongly inhibited by covalent reaction of either of two Cys residues with NEM (21–23). The reaction is ATP-protectable, and the target residues were shown to correspond to Cys-431 and Cys-1074, lying in the Walker A sequences of NBS1 and NBS2, respectively. These were the only Cys residues apparently exposed for reaction out of seven total Cys residues in hamster or human Pgp. None of the Cys residues in human MDR1 Pgp are essential because a Cys-less construct in which all Cys were changed to Ala retains drug-stimulated ATPase and drug transport activities (22, 24). In a recent report (25), we demonstrated that mouse MDR3 Pgp can be obtained in pure soluble form in large quantity after expression in Pichia pastoris, followed by solubilization in detergent, nickel affinity chromatography, and ion-exchange chromatography. Purified mouse MDR3 Pgp showed considerable activation of ATPase (5–8-fold) upon pre-incubation with DT, and we attributed this effect to cleavage of an inhibitory disulfide bond present in purified Pgp. As discussed previously (25), this finding is of interest because it could be part of a physiological regulatory mechanism involving disulfide bond formation, similar to that believed to operate in vacuolar ATPases (26, 27), and it might lead to new ways to inhibit Pgp in vivo. Therefore, it was important to establish whether the same inhibitory effects of disulfide cross-linking were seen in the human protein. As noted earlier (25), it

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¶ The abbreviations used are: Pgp, P-glycoprotein; NEM, N-ethylmaleimide; DT, dithiothreitol; bp, base pair(s).

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* Residue numbers refer to human MDR1 Pgp throughout.
seemed likely that the inhibitory disulfide bond(s) involved either or both of the Walker A Cys residues. Therefore, determination of the exact location could also shed light on the mode of interaction of the two nucleotide-binding sites. In a series of studies, we (14, 17, 18, 20) and others (28) have demonstrated that both of the nucleotide-binding sites in Pgp must be intact and must interact for catalysis to occur.

The first goal of this study was to devise a method for expression and purification of large amounts of pure human wild-type and Cys-less MDR1 Pgp using P. pastoris, for this work and future biochemical, biophysical, and high resolution structural analysis. The second goal was to determine whether inhibitory disulfide cross-linking occurs in human wild-type Pgp, to establish the location of the inhibitory disulfide bond(s), and to draw conclusions regarding the structural juxtaposition of the two nucleotide-binding sites.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmid Containing Human Wild-type MDR1 cDNA—**Human wild-type MDR1 cDNA was excised from pVTMDR (29) with HindIII and Xhol and cloned into the corresponding sites of pBluescript II (Stratagene). Full-length MDR1 DNA including ~20 bp of 5’-untranslated sequence and ~300 bp of 3’-untranslated sequence was excised from pBlMDR1 with EcoRI and Xhol, blunt-ended with T4 DNA polymerase, and ligated into pHIIL-D2 (30) cut with EcoRI and blunt-ended with T4 DNA polymerase to create pHIL-hMDR1. Initial tests showed that expression of Pgp, after transformation of this construct into P. pastoris, was low. Therefore, the 5’-end was modified to improve expression by introducing the sequence AAAAAA immediately upstream of the ATG initiation codon and removal of 5’- and 3’-untranslated sequences (31). For this, a standard polymerase chain reaction protocol (32) was used. The forward oligonucleotide was 5’-CCGC-5’ and the reverse oligonucleotide was 5’-CACTAGTCAAGAAGAGCTTCC-3’, which were used after the 5’-end was modified as follows. After application of the “mixture” consisting of 60% (v/v) P. pastoris mixture and 40% (v/v) P. pastoris cells, Pgp proteins had a 10-His tag, the purification procedure of Ref. 33 was modified as follows. After application of the n-dodecyl β-D-maltoside-solubilized supernatant to the Ni2+-nitrotriacetic acid column and washing with buffer containing 20 m M imidazole, Pgp was eluted with buffer containing 300 m M imidazole. The eluate was then diluted with 3 volumes of buffer B (25) before application to the DE52 column. Pgp was eluted with buffer B containing 1 m M 2-mercaptoethanol (25), in this way, it was omitted.

**Expression of Pgp in P. pastoris—** Pgp proteins encoded by the pHIL plasmids constructed above were expressed in P. pastoris as described (20, 25, 30, 33). Purification of Pgp—Cells were grown in flask culture, and cell disruption was performed, preparation of crude microsomes, and solubilization with n-dodecyl-β-D-maltoside as described. 3 m M Pgp proteins had a 10-His tag, the purification procedure of Ref. 33 was modified as follows. After application of the n-dodecyl β-D-maltoside-solubilized supernatant to the Ni2+-nitrotriacetic acid column and washing with buffer containing 20 m M imidazole, Pgp was eluted with buffer containing 300 m M imidazole. The eluate was then diluted with 3 volumes of buffer B (25) before application to the DE52 column. Pgp was eluted with buffer B containing 1 m M 2-mercaptoethanol (25), in this way, it was omitted.

**Activation of Pgp ATPase with DTT and Lipid—** This was done as described (25, 33). Briefly, Pgp was incubated with 8 m M DTT and then added 1% (w/v) Escherichia coli lipids (acetone/ether preparation; final lipid/protein ratio = 100:1. Avanti Polar Lipids, Inc.) or 1% (w/v) lipid mixture consisting of 60% E. coli lipids, 17.5% phosphatidylcholine, 10% phosphatidylethanolamine, and 12.5% cholesterol (34, 35) for 20 min at room temperature, followed by sonication for 30 s at 4 °C in a bath sonicator. Assays of ATPase Activity—** Assays of ATPase activity, including K_m values and vanadate inhibition, were as described (33).

**Reaction of ATPase Activity After Inhibition by Vanadate, Photoaffinity Labeling with 8-Azido-α,α’-PATP, and Vanadate-induced Trapping of Nucleotide with 8-Azido-α,α’-PATP—** These experiments were performed as described (33).

**Induction of Disulfide Cross-links by Cu2+ Oxidation—** Pgp (60 μg) was incubated with 150 m M DTT for 20 min at 20 °C and then passed through a centrifuge column in 50 m M Tris-Cl, pH 7.5, 10% glycerol, and 0.001% n-dodecyl β-D-maltoside. 5-μg samples were incubated with or without 5 μg of Cu2+ (7.5 m M) for 15 min at 37 °C. The reaction was stopped by addition of EDTA (40 m M). All samples were then incubated for an additional 10 min at 37 °C either with or without 100 m M EDTA. Samples were depolymerized for SDS gel electrophoresis as described below, except DTT was omitted. For modifications to this procedure, see the legends to the figures and tables.

**Routine Procedures—** Protein concentrations were determined by the method of H. Folin and co-workers (24), SpeI (bp 1694 and 2294), SmalI (bp 2048), AflII (bp 3215), BamHI (bp 3632), AvrII (bp 3759), and SfiI (bp 4025). The entire segment modified was sequenced to ascertain the presence of the Cys-to-Ala mutations, the presence of all restriction sites, and the absence of any undesired mutations. The final construct contained in plasmid pHIL-CL1.9-His10 gave excellent expressivity of human Cys-less MDR1 protein in P. pastoris.

**Construction of Plasmids for Expression of CL-3C and CL-4C Proteins—** Plasmid pHIL-CL3C was constructed by moving a 633-bp BamHI-SnaBI fragment (positions 3623-4265) from pHIL-MDR1.4-His10 into pHIL-CL1.9-His10. Confirmation of the correct construct was performed by sequencing the Pgp site (position 3707), digestion for Cys-1125, and the absence of a Hhal site (position 3644), diagnostic for Cys-1074. Plasmid pHIL-CL4C was constructed by moving a 600-bp SpeI-SpeI fragment (positions 1894-2294) from pHIL-MDR1.4-His10 into pHIL-CL3C. Confirmation of the correct construct was shown by the absence of a Hhal site (position 1715), diagnostic for Cys-431.

**Introduction of Natural Cys-431, Cys-1074, and Cys-431/Cys-1074 into Cys-less MDR1 Pgp—** For purification of Pgp containing the single Cys-431 residue, a plasmid was constructed by moving a 600-bp SpeI-SpeI fragment (positions 1694-2294) from pHIL-MDR1.4-His10 into pHIL-CL1.9-His10 to create pHIL-C431. A plasmid for Pgp containing the single Cys-1074 residue was constructed by site-directed mutagenesis using the Altered Sites II kit. The template consisted of a KpnI-BamHI fragment (base pairs 3199-4313) of pHIL-1.9 (analogous to pHBlMDR1-6A10His above) ligated into pALT1ER to create template pALT-CL-C. In addition to its Cys-to-Ala mutations, pALT-CL-C contains engineered AflI (base 3215), BamHI (base 3632), AvrII (base 3759), and SfiI (base 4025) sites. The mutagenic oligonucleotide was GGATCCGACGTCTGGAAGACGACAGC, where the underlined bases indicated replacement of Ala (GCT) with Cys (TCT) and the destruction of a Hhal site. The mutation was transferred to pHIL-CL1.9 on an AflI-AvrII fragment after sequencing to verify the presence of Cys-1074 and the absence of undesired mutations. The new plasmid was checked by digestion with Hhal and was named pHIL-C1074. A plasmid for Pgp containing both Cys-431 and Cys-1074 was constructed by moving an AflI-AvrII fragment from pHIL-C1074 into pHIL-C431. All pHIL plasmids were checked by digestion with a combination of SacI, SpeI, SfiI, and XhoI to ensure that the overall size was correct.

Assays of ATPase Activity—** Assays of ATPase activity, including K_m values and vanadate inhibition, were as described (33).
bicinchoninic acid method in the presence of 1% SDS using bovine serum albumin as a standard (36). SDS-polyacrylamide gel electrophoresis was done using the Mini-PROTEAN II Gel and Electrotransfer 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, and 160 mM DTT for 20 min at 37 °C and then run on 10% polyacrylamide gels. For immunodetection of Pgp, mouse monoclonal antibody C219 (25). For expression of human MDR1 Pgp in P. pastoris and Purification to Homogeneity—It has been previously used for mouse MDR3 Pgp (25, 33) with minor modifications (pHIL-hMDR1); lane 2, cells transformed with MDR1 cDNA after 5'-modification, removal of 5'- and 3'-sequences, and addition of a 10-His tag (pHIL-hMDR1-5A10His); lane 3, same as lane 2, but after insertion of additional SpeI, BamHI, and SnaBI sites (pHIL-MDR1.4-His10); lane 4, cells transformed with pHIL-CL1.9-His10, expressing human Cys-less Pgp.

**FIG. 1. Expression of human wild-type and Cys-less MDR1 P-glycoproteins in P. pastoris.** Transformed P. pastoris cells showing the His "Mut" phenotype (34) were grown in 10 ml of culture with methanol induction; a "rapid membranes preparation" was made; and 15 μg were protein run on SDS gels and immunoblotted with monoclonal antibody C219 (25). Construction of the plasmids and methods for expression of the constructs are described under "Experimental Procedures." Lane 1, cells transformed with MDR1 cDNA before modifications (pHIL-hMDR1); lane 2, cells transformed with MDR1 cDNA after 5'-modification, removal of 5'- and 3'-sequences, and addition of a 10-His tag (pHIL-hMDR1-6A10His); lane 3, same as lane 2, but after insertion of additional SpeI, BamHI, and SnaBI sites (pHIL-MDR1.4-His10); lane 4, cells transformed with pHIL-CL1.9-His10, expressing human Cys-less Pgp. A series of P. pastoris cells showing human wild-type and Cys-less Pgp, activation was seen with lipid, but DTT had no effect. Two kinds of lipid were used, namely E. coli lipids or a lipid mixture designed to approximately mimic mammalian plasma membrane lipids (34, 35), and they gave similar results. (Other lipids, e.g. asolectin, also gave similar data.) Table I summarizes the catalytic properties of the activated human wild-type and Cys-less MDR1 proteins. The specific ATPase activities of wild-type and Cys-less Pgp were similar to those previously reported (38), with Cys-less Pgp having ~60% of wild-type activity. Inclusion of lipids at 0.1 or 0.2% (w/v) in solubilization and chromatography buffers during purification had no effect on final specific ATPase activities. There was low "basal" activity in the absence of drug (Table I), which was greatly accelerated by verapamil, demonstrating that we are dealing with physiologically relevant activity. K_i values for wild-type and Cys-less Pgp were similar to each other (Table I) and to published data (35). We performed experiments in which ATPase activity was measured as a function of increasing verapamil concentration, from which the apparent K_i for verapamil (concentration for 50% stimulation of activity) was calculated (Table I), showing that Cys-less Pgp consistently had the lower apparent K_i. Vanadate fully inhibited the ATPase activity of wild-type and Cys-less Pgp; from titrations, the IC_50 values were found to be similar (Table I). Reactivation after vanadate inhibition occurred at almost identical rates (Table I). Further experiments (not shown) indicated that Mg-8-azido-ATP binding and vanadate trapping of nucleotide Mg-8-azido-ADP were similar in human Cys-less and wild-type Pgp. In summary, we have successfully expressed human wild-type and Cys-less pro-

**RESULTS**

Expression of human Wild-type and Cys-less P-glycoproteins in P. pastoris and Purification to Homogeneity—It has been demonstrated that mouse MDR3 Pgp can be expressed in P. pastoris (37) and purified in far higher amount than from any other system (25). For expression of human MDR1 Pgp in P. pastoris, human MDR1 cDNA was modified and incorporated into plasmid pHIL-D2 (30). Details of these manipulations are given under "Experimental Procedures." Fig. 1 (lane 1) shows that expression of human Pgp from the initial construct (pPHIL-hMDR1) was poor. After introduction of 5'-AAAAA, removal of 5'- and 3'-untranslated sequences, and addition of the C-terminal His tag, expression of human MDR1 Pgp was much improved (lane 2). Addition of restriction sites did not affect the level of expression (lane 3).

For purification of human MDR1 Pgp, cells were grown in flask culture with methanol induction (see "Experimental Procedures"). The method for purification followed in general that previously used for mouse MDR3 Pgp (25, 33) with minor modifications (see "Experimental Procedures"). The final MDR1 Pgp protein showed a single 140-kDa band on SDS gels in the presence or absence of reducing agent at 140 kDa, showing that it is deficient in normal glycosylation; the yield of pure soluble MDR1 Pgp protein showed a single band on SDS gels in the presence or absence of reducing agent. Thus, from 6 liters of cells, the yield of purified soluble Cys-less protein was the same as for wild-type MDR1 Pgp (see above). An SDS gel of the pure protein showed a single 140-kDa band in the presence or absence of reducing agent.

**Characterization of ATPase Activity and Nucleotide Binding Properties of Human Wild-type and Cys-less MDR1 Pgp Purified from P. pastoris**—We showed previously that the ATPase activity of detergent-soluble pure mouse MDR3 Pgp obtained after expression in P. pastoris was significantly activated by both DTT and lipid (25). This was found here to be the case with human wild-type MDR1 Pgp. The degree of activation by DTT was 2–3-fold, which is less than that seen with mouse MDR3 Pgp (5–8-fold), but still significant. With human Cys-less Pgp, activation was seen with lipid, but DTT had no effect. Two kinds of lipid were used, namely E. coli lipids or a lipid mixture designed to approximately mimic mammalian plasma membrane lipids (34, 35), and they gave similar results. (Other lipids, e.g. asolectin, also gave similar data.) Table I summarizes the catalytic properties of the activated human wild-type and Cys-less MDR1 proteins. The specific ATPase activities of wild-type and Cys-less Pgp were similar to those previously reported (38), with Cys-less Pgp having ~60% of wild-type activity. Inclusion of lipids at 0.1 or 0.2% (w/v) in solubilization and chromatography buffers during purification had no effect on final specific ATPase activities. There was low "basal" activity in the absence of drug (Table I), which was greatly accelerated by verapamil, demonstrating that we are dealing with physiologically relevant activity. K_i values for wild-type and Cys-less Pgp were similar to each other (Table I) and to published data (35). We performed experiments in which ATPase activity was measured as a function of increasing verapamil concentration, from which the apparent K_i for verapamil (concentration for 50% stimulation of activity) was calculated (Table I), showing that Cys-less Pgp consistently had the lower apparent K_i. Vanadate fully inhibited the ATPase activity of wild-type and Cys-less Pgp; from titrations, the IC_50 values were found to be similar (Table I). Reactivation after vanadate inhibition occurred at almost identical rates (Table I). Further experiments (not shown) indicated that Mg-8-azido-ATP binding and vanadate trapping of nucleotide Mg-8-azido-ADP were similar in human Cys-less and wild-type Pgp. In summary, we have successfully expressed human wild-type and Cys-less pro-

**TABLE I**

| Specific ATPase activity | Wild-type Pgp | Cys-less Pgp |
|--------------------------|--------------|-------------|
| (μmol/min/mg)            |              |             |
| Minus verapamil          | 0.06         | 0.08        |
| Plus 150 μM verapamil    | 1.7 (28X)    | 0.96 (12X)  |
| K<sub>i</sub> (MgATP) (> 150 μM verapamil; mM) | 1.5          | 1.2         |
| K<sub>app</sub> (μM)     |              |             |
| E. coli lipids           | 13           | 17          |
| Lipid mixture           | 0.7          | 1.5         |
| IC<sub>50</sub> (for vanadate; μM) | 2.3         | 7.4         |
| t<sub>1/2</sub> (min)    | 56           | 57          |

*In the Cys-less protein, all seven naturally occurring Cys residues were mutated to Ala (see “Experimental Procedures”).

*Values represent ATPase activity in 10 mM MgATP after activation with lipid and DTT as described under “Experimental Procedures.” Similar values were seen with E. coli lipids or in the lipid mixture. Cys-less protein had the same activity whether DTT was present or not during activation.

*Values in parentheses indicate fold stimulation by verapamil.

*Lipids are described under “Experimental Procedures.”

*Values represent concentration for 50% inhibition of ATPase.

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**Human Cys-less Pgp, in which all seven natural Cys residues were mutated to Ala, was previously constructed, expressed, and purified on small scale (~10 μg of purified protein) (24, 38). As described under “Experimental Procedures,” we modified this construct to enhance expression in P. pastoris. A series of nine new restriction sites was also inserted to facilitate future mutation experiments. The final cDNA construct (pPHIL-CL1.9-His10) was found to be expressed to the same level as wild-type MDR1 in P. pastoris (Fig. 1, lane 4), and the yield of purified soluble Cys-less protein was the same as for wild-type MDR1 Pgp (see above). An SDS gel of the pure protein showed a single 140-kDa band in the presence or absence of reducing agent.
Pgp was pre-activated with the lipid mixture without DTT (see “Experimental Procedures”). Where indicated, samples were treated with or without 1 mM Cu²⁺ (phenanthroline) for 15 min at 37 °C, followed by treatment with or without 100 μM DTT for 10 min on ice. ATPase activity was then assayed. NEM samples were pretreated with 100 μM NEM for 10 min at 37 °C. Activities are expressed relative to a sample of Pgp assayed without any treatment.

**TABLE II**

| Pgp          | Relative ATPase activity | %     | 51 | 6° |
|--------------|--------------------------|-------|----|----|
| Wild-type    | -Cu²⁺, -DTT              | 28    | 11 |    |
| Cys-less     | +Cu²⁺, +DTT              | 95    | 103| 104| 109|

* Under conditions of full inhibition of ATPase the stoichiometry of labeling of wild-type Pgp by NEM was previously shown to equal 2 mol/mol of Pgp (21, 22).

Effect of Disulfide Bond Formation on ATPase Activity—

Table II confirms that human wild-type Pgp is activated by DTT and shows that the activity was strongly reduced by incubation with Cu²⁺ and partly reversed by DTT after Cu²⁺. For comparison, experiments with NEM are included to show that it also gave strong inhibition of purified soluble wild-type Pgp. In sharp contrast, Cys-less Pgp was unaffected by DTT, Cu²⁺, or NEM. These data support the conclusion that formation of an inhibitory disulfide bond(s) in human wild-type Pgp reduces its ATPase activity.

**Fig. 2** shows a Coomassie Blue-stained SDS gel of wild-type protein treated in various ways. The gel was run without any reducing agent in the polymerizing SDS sample buffer or gel buffer. *Lane 1* shows that when purified Pgp was dissolved in sample buffer directly, a major band at 140 kDa corresponding to Pgp was seen, with slower moving components at ~200 and ~300 kDa. This demonstrates spontaneous formation of higher molecular mass species in the absence of a reducing agent. After activation by DTT and subsequent removal of DTT by passage through a centrifuge column, most of the protein ran at 140 kDa (*lane 2*). Treatment with Cu²⁺ converted most of the Pgp to the slower moving species (*lane 3*), and DTT after Cu²⁺ reversed the effect (*lane 4*). Prior treatment with NEM (*lanes 6 and 7*) prevented the effect of Cu²⁺. From these data, we can see clearly that wild-type Pgp is prone to form higher molecular mass species that involve disulfide cross-links. Preincubation with MgATP and vanadate (*lane 8*) or inclusion of MgATP (*lane 9*) gave minor protection from Cu²⁺-induced cross-linking; this, together with the results with NEM, implicates the Walker A Cys residues (Cys-431 and/or Cys-1074) of NBS1 and NBS2 as possible loci of disulfide bond formation. In *Fig. 2B*, it is shown that Cys-less Pgp did not form higher molecular mass species and ran as a single band at 140 kDa under all conditions. (The faint band seen above the 292-kDa molecular mass marker is the location of the stacking gel boundary.)

The location of higher molecular mass species in the wild type at ~200 and ~300 kDa is consistent with intra- or intermolecular disulfide bond formation. With Cu²⁺, very high molecular mass aggregates were also seen at the top of the gel, which presumably formed after the initial disulfide bond because NEM prevented their occurrence. To further elucidate the loci of disulfide bond formation, a series of proteins containing varying combinations of naturally occurring Cys residues was studied.

**Construction of CL-3C and CL-4C Proteins**—Human wild-type Pgp contains seven natural Cys residues, including three in the membrane domains, one in NBS1 (Cys-431 in the Walker A sequence), and three in NBS2 (Cys-1074 in the Walker A sequence plus Cys-1125 and Cys-1227). We constructed, expressed, and purified CL-3C protein, containing just the three natural Cys residues in NBS2, and CL-4C protein, containing the three natural Cys residues in NBS2 plus the single natural Cys residue in NBS1 (i.e. neither CL-3C nor CL-4C contains any membrane domain Cys). The yield and purity of the two proteins were as for the wild type. After activation by lipid and DTT, both showed verapamil-stimulated ATPase, with the following specific activities: 1.2 and 1.1 μmol/mg/min for CL-4C and CL-3C, respectively. CL-4C showed 2.4-fold activation by DTT, similar to the wild type, whereas CL-3C showed only 1.17-fold activation by DTT. These data are shown in Table III. The proteins were treated and run on SDS gels following the same protocols as described for *Fig. 2 (A and B)*. The results are shown in *Fig. 3 (A, CL-4C; and B, CL-3C)*. It is shown in *Fig. 3A* that CL-4C behaved similarly to the wild type in *Fig. 2A*. With Cu²⁺, the 200-kDa band was pronounced (*lane 3*); the ~300-kDa band was also present (*lane 3*); and there was significant protection by the catalytic site ligand MgATP plus vanadate or MgATP alone (*lanes 8 and 9*). These data clearly show that the membrane domain Cys residues (which are absent from CL-4C) are not involved in the formation of the 200- or ~300-kDa cross-linked bands. In contrast, CL-3C in *Fig. 3B* behaved similarly to Cys-less Pgp in *Fig. 2B*. In CL-3C, Cu²⁺ did not appear to cause formation of cross-links to anywhere near the same extent as in CL-4C. These results implicate Cys-431 in NBS1 as necessary for formation of the inhibitory disulfide bond(s).

**Experiments with Cys-431, Cys-1074, and Cys-431/Cys-1074 Proteins**—For further studies, we constructed, expressed, and purified proteins containing either Cys-431 or Cys-1074 or both Cys-431 and Cys-1074. The yield and purity of the three proteins were as for the wild type. All showed verapamil-stimulated ATPase, with the following specific activities: 1.3, 0.82, and 1.5 μmol/mg/min for Cys-431/Cys-1074, Cys-431, and Cys-1074, respectively. Cys-431/Cys-1074 Pgp was activated 2.00-fold by DTT; Cys-1074 Pgp was activated by 1.17-fold; and Cys-431 Pgp showed only 1.02-fold activation (see Table III). The proteins were then treated exactly as described for *Fig. 2*, and the results are shown in *Fig. 4*. Cys-431/Cys-1074 Pgp (*Fig. 4 A*) behaved in a manner similar to the wild type (*Fig. 2A*) and CL-4C (*Fig. 3A*) in that 200- and ~300-kDa bands were seen in the absence of DTT or after Cu²⁺ treatment. After cross-linking with Cu²⁺, the band at 200 kDa was pronounced and sharp (*Fig. 4A, lane 3*). With the Cys-431/Cys-1074 protein, significant protection by MgATP plus vanadate or by MgATP alone was seen, and NEM protected strongly. The results leave no doubt that a disulfide bond forms and suggest that the 200-kDa band represents an intramolecularly disulfide-cross-linked product between Cys-431 and Cys-1074. It may be that the less sharp nature of this band in the wild type and CL-4C (*Figs. 2A and 3A*) is due to the fact that Cys-1125 and Cys-1227
in NBS2 affect the cross-linking pattern, perhaps by also cross-linking to Cys-431, to produce species of slightly different mobility and hence a more diffuse band at ~200 kDa; however, we did not examine this question further.

The results for Cys-431 Pgp were quite different (Fig. 4B). Here, no higher molecular mass species were seen in lanes 1 and 2 (omission of DTT), and Cu$^{2+}$ treatment produced a single band at 300 kDa only, with no band at 200 kDa (lane 3). This was due to disulfide bond formation since it was reversed by DTT (lane 4) and prevented by pretreatment with NEM (lanes 6 and 7). The results for Cys-1074 Pgp (Fig. 4C) also showed no cross-linking in lanes 1 and 2 (omission of DTT) and formation of two higher molecular mass bands with Cu$^{2+}$, a major one at 290 kDa and a minor one at 300 kDa. Again, these bands were products of disulfide cross-linking since they were reversed by DTT and prevented by NEM. No band at 200 kDa was evident. These results suggest that the cross-linked bands at ~290–300 kDa are due to intermolecular cross-links (i.e. Pgp dimers) and support the idea that the 200-kDa band in the wild-type, CL-4C, and Cys-431/Cys-1074 proteins is an intramolecularly cross-linked species.

Gradient SDS Gel Electrophoresis to Further Define Size of Cross-linked Species—Fig. 5 shows a 4–20% gradient polyacrylamide gel of all the proteins after Cu$^{2+}$ treatment for direct comparison and better determination of the sizes of the cross-linked species. It is confirmed that the wild-type (hMDR1), CL-4C, and Cys-431/Cys-1074 proteins behaved similarly, producing species at 200 and 300 kDa. The Cys-431 and Cys-1074 proteins did not show any band at 200 kDa, but did show distinct bands at 290 and 300 kDa, probably of slightly different shape due to the different cross-links. The CL-3C and Cys-less proteins showed only the non-cross-linked Pgp band at 140 kDa. (Traces of the 300-kDa band were present in CL-3C.)

Limited Trypsin Digestion of Cross-linked Pgp—Limited trypsin digestion of human wild-type MDR1 Pgp (lacking glycosylation) yields two fragments, the N-terminal half of apparent 70 kDa and the C-terminal half of apparent 60 kDa (16). If the 200-kDa species seen in Cys-431/Cys-1074 Pgp after Cu$^{2+}$ treatment is due to formation of an intramolecular disulfide bond between Cys-431 in NBS1 and Cys-1074 in NBS2, then, in theory, trypsin digestion of the cross-linked species should produce no N- or C-terminal half fragments. Also, if the cross-linked band at 300 kDa in the Cys-431 protein is due to a dimer of Pgp formed by an intermolecular Cys-431–Cys-431 disulfide bond, then trypsin digestion should produce only C-terminal half fragments. Finally, if the bands at 290 and 300 kDa in the cross-linked Cys-1074 protein are due to Pgp dimers formed by intermolecular Cys-1074–Cys-1074 disulfide bonds, then tryptic digestion should produce N-terminal (but no C-terminal) half fragments.

Fig. 6A shows the experiment with the Cys-less and Cys-431/Cys-1074 proteins. It is seen that the Cys-less protein, after Cu$^{2+}$ treatment and limited trypsin digestion, showed both N- and C-terminal half bands. Notably, after Cu$^{2+}$ treatment and trypsin digestion, the Cys-431/Cys-1074 protein did not show these bands. (The faint bands seen are presumably derived from residual uncross-linked Pgp.) This provides confirmation that an intramolecular disulfide bond forms between Cys-431 and Cys-1074 and generates the 200-kDa band.

Fig. 6B shows the results for Cys-431 Pgp. Unfortunately, there was a significant amount of residual uncross-linked Pgp after Cu$^{2+}$ treatment, which was always seen (Figs. 4 and 5) and which obscures the results because it generated both N- and C-terminal half fragments. However, it is shown that after Cu$^{2+}$ and trypsin treatment, the ratio of the two fragments was greatly in favor of the 60-kDa fragment (C-terminal half); and therefore, the result confirms that the cross-linked band after Cu$^{2+}$ treatment of Cys-431 Pgp seen in Fig. 5 is a Pgp dimer.
produced by a Cys-431–Cys-431 disulfide bond. The results for Cys-1074 Pgp in Fig. 6B also favor the conclusion that the high molecular mass bands seen after Cu²⁺ treatment (Fig. 5) contain intermolecularly cross-linked species because after trypsin treatment, the N-terminal half fragment was consistently seen to be present in greater amounts than the C-terminal half fragment. Again, the presence of significant residual uncross-linked Pgp after Cu²⁺ treatment meant that some C-terminal half fragment was always seen in the experiment.

**Experiments with Hamster Pgp in Plasma Membranes**—Plasma membranes containing hamster Pgp in its natural membrane environment were prepared as described (36). The molecular size of Pgp in these membranes is ~170 kDa due to extensive natural glycosylation. Oxidation with Cu²⁺ was carried out on the membranes directly as described above for pure soluble human wild-type Pgp, and it was seen (by SDS gel electrophoresis and immunoblotting) that higher molecular mass species were formed that corresponded to the same bands seen with human Pgp, after allowance for extra mass due to glycosylation was made (data not shown). Cross-linking with
Cu\textsuperscript{2+} reduced the ATPase activity of membranous hamster Pgp by 89%, exactly the same as with human wild-type Pgp in Table II. Therefore, the environment of Pgp (membrane versus detergent-soluble) did not change the effects of Cu\textsuperscript{2+} cross-linking.

**DISCUSSION**

Human wild-type MDR1 P-glycoprotein was expressed in *P. pastoris* and purified to homogeneity in detergent-soluble form in high yield following a procedure previously developed for mouse MDR3 Pgp (25, 33). 6 liters of flask culture of cells yielded 4.5 mg of pure MDR1 protein, which showed strong verapamil-stimulated ATPase activity after activation by lipid and DTT. Human Cys-less Pgp, in which all seven Cys residues were changed to Ala, was purified in similar yield by the same procedure. It had similar verapamil-stimulated ATPase activity to that reported in previous studies. This work therefore provides a new method for high yield purification of human Pgp in detergent-soluble form for biochemical, biophysical, and high resolution structural studies. Larger scale preparations (~100 mg) may be achieved readily by fermentor culture (25) if required.

DTT activated the ATPase of wild-type MDR1 Pgp by ~2-fold, and we suspected this was due to the presence of inhibitory disulfide bond(s). MDR1 Pgp ran as a single 140-kDa band on SDS gels in the presence of reducing agent, but showed spontaneous formation of higher molecular mass bands in the absence of reducing agent. Cys-less Pgp showed no spontaneous formation of higher molecular mass species or any activation of ATPase by DTT. Oxidation of wild-type Pgp with Cu\textsuperscript{2+} (phenanthroline) generated the same higher molecular mass species (predominantly at 200 and ~300 kDa) that formed spontaneously in the absence of reducing agent. With Cu\textsuperscript{2+}, cross-linking was much more complete, and subsequent treatment with DTT reduced the Pgp back to a single 140-kDa band. On the other hand, Cys-less Pgp was unaffected by Cu\textsuperscript{2+} treatment. Using proteins constructed to contain specific combinations of naturally occurring Cys residues, we demonstrated that the two Walker A Cys residues (Cys-431 in NBS1 and Cys-1074 in NBS2) were involved in disulfide bond formation. Two distinct types of cross-linked Pgp species were formed. One type, running with a molecular mass of 290–300 kDa on SDS gels, consisted of dimeric Pgp species formed by an intermolecular disulfide bond between Cys-431 and Cys-431 or between Cys-1074 and Cys-1074. (The role of additional dimer-forming disulfides involving Cys-1125 and Cys-1227 in NBS2 cannot be ruled out.) The second type consisted of a 200-kDa band and was due to formation of an intramolecular cross-link between Cys-431 in NBS1 and Cys-1074 in NBS2. This species was particularly prominent in the Cys-431/Cys-1074 protein.

The ATPase activity of proteins containing both Cys-431 and Cys-1074 (i.e. CL-4C and Cys-431/Cys-1074) was activated by DTT to about the same extent as the wild type, whereas proteins in which only one of these two Cys residues was present (single Cys-431, single Cys-1074, or CL-3C) were activated to little or no extent. Thus, the major inhibitory disulfide is the intramolecular one that forms between Cys-431 and Cys-1074, and the degree of DTT activation correlates with spontaneous formation of this species.

Results with hamster Pgp in plasma membranes confirmed that disulfide cross-linking occurred after Cu\textsuperscript{2+} oxidation and that it resulted in inhibition of ATPase activity. We had long been aware that storage of plasma membranes in the absence of reducing agent leads to loss of activity of hamster Pgp ATPase, which can be fully restored by DTT. Thus, disulfide cross-linking occurs in the natural membrane environment. One major conclusion from this work is that the two nucleo-
Disulfide Cross-linking of P-glycoprotein Nucleotide Sites

for excellent technical assistance.

quires that the two Walker A sequences approach to within a bound nucleotide. Disulfide bond formation between them requires that the two Walker A sequences approach to within a few angstroms of each other. (The Cα–Cα distance of two cross-linked Cys residues is 4.4–6.8 Å (39).) Therefore, the structural arrangement of the two Pgp nucleotide-binding sites is likely to resemble that seen in the recent x-ray structure of ArsA (40), in which the two nucleotide sites are close, and the two domains are interdigitated. Conversely, the dimeric HisP x-ray structure, showing an arrangement in which the two bound ATP are very far apart (41), is unlikely to represent the structure found in Pgp. Possibly, in the absence of the membrane domains, the two HisP subunits were forced into an unnatural “back-to-back” configuration by crystal packing forces. Current data and concepts regarding the catalytic mechanism of Pgp seem consistent with (even to require) intimate structural juxtaposition of the ATP-binding sites, as discussed previously (33).

A second conclusion from this work is that an in vivo regulatory mechanism for controlling Pgp activity, similar to that proposed for vacuolar ATPase (26, 27) involving formation of an inhibitory disulfide cross-linking event, could be artificially induced in cancer cells, this could be a mechanism to inhibit human Pgp in situ.

In a previous report (42), inhibitory disulfide cross-linking was detected between the natural Cys-1074 residue in NBS2 of human Pgp and the engineered mutant residues Cys-427 (mutation G427C) and Cys-439 (mutation L439C) in NBS1. In contrast to results reported here, no disulfide cross-linking was seen between the natural Cys-431 residue in NBS1 and Cys-1074 in NBS2 (42). At the present time, it is unclear why this difference occurred since experimental conditions were generally similar. On the basis of the results, it was concluded in the previous report (42) that the two predicted nucleotide sites in Pgp are close to each other, a conclusion with which we are in agreement. The work carried out here, using only the natural Cys residues in both pure soluble and plasma membrane-located proteins, emphasizes that this is the case in the natural environment and that it can lead to inhibition by disulfide bond formation in the wild-type protein.

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REFERENCES

1. Gottesman, M. M., and Pastan, I. (1993) Annu. Rev. Biochem. 62, 385–427
2. Sharom, F. J. (1997) J. Membr. Biol. 160, 161–175
3. Stein, W. D. (1997) Physiol. Rev. 77, 545–590
4. Ambudkar, S. V., Dey, S., Hrycyna, C. A., Ramachandra, M., Pastan, I., and Gottesman, M. M. (1999) Annu. Rev. Pharmacol. Toxicol. 39, 361–398
5. Kim, R. B., Fromm, M. F., Wandel, C., Leake, B., Wood, A. J. J., Roden, D. M., and Wilkinson, G. R. (1998) J. Clin. Invest. 101, 289–294
6. Lee, C. G. L., Gottesman, M. M., Cardarelli, C. O., Ramachandra, M., Jeang, K.-T., Ambudkar, S. V., Pastan, I., and Dey, S. (1999) Biochemistry 37, 3594–3601
7. Bruggeman, E. P., Carrier, S. J., Gottesman, M. M., and Pastan, I. (1992) J. Biol. Chem. 267, 21020–21026
8. Zhang, T. J., Collins, K. I., and Greenberger, L. M. (1995) J. Biol. Chem. 270, 5441–5448
9. Demeule, M., Laplante, A., Murphy, G. F., Wenger, R. M., and Beliveau, R. (1998) Biochemistry 37, 18110–18118
10. Demmer, A., Andreue, S., Thole, H., and Tummerl, B. (1999) Eur. J. Biochem. 264, 800–805
11. Loo, T. W., and Clarke, D. M. (1999) J. Biol. Chem. 274, 35388–35392
12. Senior, A. E., Al-Shawi, M. K., and Urbatsch, I. L. (1995) FEBS Lett. 377, 585–589
13. Urbatsch, I. L., Sankaran, B., Weber, J., and Senior, A. E. R. (1995) J. Biol. Chem. 270, 19383–19390
14. Urbatsch, I. L., Sankaran, B., Bhagat, S., and Senior, A. E. (1995) J. Biol. Chem. 270, 2656–2661
15. Sauna, Z. E., and Ambudkar, S. V. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2515–2520
16. Dey, S., Ramachandra, M., Pastan, I., Gottesman, M. M., and Ambudkar, S. V. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10594–10599
17. Sankaran, B., Bhagat, S., and Senior, A. E. (1997) Biochemistry 36, 6847–6853
18. Senior, A. E., and Bhagat, S. (1998) Biochemistry 37, 831–836
19. Szabó, K., Welker, E., Bakos, E., Müller, M., Roninson, I., Varadi, A., and Sarkadi, B. (1998) J. Biol. Chem. 273, 10132–10138
20. Urbatsch, I. L., Beaudet, L., Carrier, I., and Gros, P. (1998) Biochemistry 37, 4592–4602
21. Al-Shawi, M. K., Urbatsch, I. U., and Senior, A. E. (1994) J. Biol. Chem. 269, 8986–8992
22. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 22957–22961
23. Urbatsch, I. L., Al-Shawi, M. K., and Senior, A. E. (1994) Biochemistry 33, 7069–7076
24. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 843–848
25. Lerner-Marmarosh, N., Gimi, K., Urbatsch, I. U., Gros, P., and Senior, A. E. (1997) J. Biol. Chem. 272, 34711–34718
26. Forgac, M. (1999) J. Biol. Chem. 274, 1301–1305
27. Oluwatson, Y. E., and Kane, P. M. (1997) J. Biol. Chem. 272, 28149–28157
28. Hrycyna, C. A., Ramachandra, M., Ambudkar, S. V., Ko, Y. H., Pedersen, P. L., Urbatsch, I. L., and Gottesman, M. M. (1998) J. Biol. Chem. 273, 16631–16634
29. Goodfellow, H. R., Sardini, A., Ruetz, S., Callaghan, R., Gros, P., McNaughton, P. A., and Higgins, F. C. (1996) J. Biol. Chem. 271, 13668–13674
30. Invitrogen (1999) Pichia Expression Kit Version J Instruction Manual, p. 2, Invitrogen, Carlsbad, CA
31. Hinnebusch, A. G., and Liebman, S. W. (1991) The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis, and Energetics, Vol. I, pp. 625–649, Cold Spring Harbor Laboratory Press, Plainview, NY
32. Innis, A., and Gelfand, D. H. (1990) in PCR Protocols (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds), pp. 3–12, Academic Press, Inc., New York, NY
33. Urbatsch, I. L., Gimi, K., Wilke-Mounts, S., and Senior, A. E. (2000) J. Biol. Chem. 275, 25031–25038
34. Ambudkar, S. V., Lelong, I. H., Zhang, J., Cardarelli, C. O., Gottesman, M. M., and Pastan, I. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8472–8476
35. Figler, R. A., Omote, H., Nakamoto, R. K., and Al-Shawi, M. K. (2000) Arch. Biochem. Biophys. 376, 34–46
36. Al-Shawi, M. K., and Senior, A. E. (1993) J. Biol. Chem. 268, 4197–4206
37. Beaudet, L., Urbatsch, I. L., and Gros, P. (1998) Methods Enzymol. 292, 397–413
38. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 21449–21452
39. Richardson, J. S. (1981) Adv. Protein Chem. 34, 167–339
40. Zhou, T., Radaev, S., Rosen, B. P., and Gatti, D. L. (2000) EMBO J. 19, 4838–4845
41. Hung, L. W., Wang, I. X., Nikaido, K., Liu, P. Q., Ames, G. F., and Kim, S. H. (1998) Nature 396, 703–707
42. Loo, T. W., and Clarke, D. M. (2000) J. Biol. Chem. 275, 19435–19438
Cysteines 431 and 1074 Are Responsible for Inhibitory Disulfide Cross-linking between the Two Nucleotide-binding Sites in Human P-glycoprotein
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