Role of GSH in Estrone Sulfate Binding and Translocation by the Multidrug Resistance Protein 1 (MRP1/ABCC1)*

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Multidrug resistance protein 1 (MRP1/ABCC1) is an ATP-dependent efflux pump that can confer resistance to multiple anticancer drugs and transport conjugated organic anions. Unusually, transport of several MRP1 substrates requires glutathione (GSH). For example, estrone sulfate transport by MRP1 is stimulated by GSH, vincristine is co-transported with GSH, or GSH can be transported alone. In the present study, radioligand binding assays were developed to investigate the mechanistic details of GSH-stimulated transport of estrone sulfate by MRP1. We have established that estrone sulfate binding to MRP1 requires GSH, or its non-reducing analogue S-methyl GSH (S-mGSH), and further that the affinity (Kd) of MRP1 for estrone sulfate is 2.5-fold higher in the presence of S-mGSH than GSH itself. Association kinetics show that GSH binds to MRP1 first, and we propose that GSH binding induces a conformational change, which makes the estrone sulfate binding site accessible. Binding of non-hydrolyzable ATP analogues to MRP1 decreases the affinity for estrone sulfate. However, GSH (or S-mGSH) is still required for estrone sulfate binding, and the affinity for GSH is unchanged. Estrone sulfate affinity remains low following hydrolysis of ATP. The affinity for GSH also appears to decrease in the post-hydrolytic state. Our results indicate ATP binding is sufficient for reconfiguration of the estrone sulfate binding site to lower affinity and argue for the presence of a modulatory GSH binding site not associated with transport of this tripeptide. A model for the mechanism of GSH-stimulated estrone sulfate transport is proposed.

The development of resistance to chemotherapy remains one of the biggest obstacles to the successful treatment of cancer. One of the mechanisms by which tumors acquire resistance is the overexpression of membrane transport proteins such as multidrug resistance protein 1 (MRP1) or ABCC1 (1–3). MRP1 is a member of the C subfamily of the ABC superfamily of transport proteins. It is expressed in most tissues in the body and utilizes energy from ATP hydrolysis to transport a wide variety of structurally and functionally distinct molecules across the plasma membrane. Many MRP1 substrates are conjugated organic anions such as LTC4 (4, 5), E217-βG (6, 7), and estrone sulfate (8). In addition to transporting these endogenously formed metabolites, MRP1 has an important role in detoxification and protection of normal tissues from xenobiotics (9–12). However, this is problematic when MRP1 is expressed at elevated levels in cancer cells, because it causes resistance to anticancer agents, including anthracyclines, plant alkaloids, and antifolates, by transporting them out of cells.

An unusual feature of MRP1 (and some other members of the ABC subfamily) is the requirement for the reducing tripeptide GSH to be present for transport of several of its substrates to occur, including certain anticancer drugs (5, 8, 13–17). GSH is an essential cellular antioxidant, which reacts with electrophilic molecules to form conjugated hydrophilic metabolites that can be more readily eliminated from the body. Prior to elimination, such metabolites must be actively effluxed from the cells in which they are formed. MRP1 can transport many such GSH-conjugated anions (18); however, the role of GSH in MRP1-mediated transport is not limited to forming conjugates. For example, transport of the Vinca alkaloid vincristine is markedly enhanced by GSH, and the interaction appears to be co-transport with the drug without formation of a conjugate (14). Estrone sulfate transport by MRP1 is also enhanced by GSH; however, in contrast to vincristine transport, GSH only stimulates the process and is not itself transported (8). Some MRP1 inhibitors such as the tricyclic isoxazole derivative LY475776 also require GSH to photo-label to MRP1 (19), yet transport of some drugs, such as the antifolate methotrexate occur independently of GSH (20). In contrast, GSH can be transported alone, and some xenobiotics, including verapamil, or bioflavonoids such as apigenin, stimulate GSH efflux without being transported themselves (21, 22).

Cellular GSH-oxidized glutathione levels are important for regulating the redox status of the cell and modulating responses to oxidative stress. Efflux of GSH in response to cell damage has been found to be part of an apoptotic signaling pathway (23–26), and it has recently been demonstrated that this efflux can be mediated by MRP1 (26, 27). However, it is not the reducing ability of GSH that is important for its interactions with MRP1, because non-reducing analogues such as ophthallic acid or S-mGSH can functionally substitute for GSH (8, 14, 16, 17, 19). Thus it is clear that the role of GSH in MRP1-mediated transport is very complex, but as yet relatively little is known about the mechanism of interaction of GSH with this transporter.

MRP1 function is often evaluated by measuring ATP-dependent uptake of a radiolabeled substrate into inside-out membrane vesicles prepared from cells expressing the transporter. Transmembrane transport of a substrate assayed in this way gives an overall measure of a multistep process. First, the substrate binds to a high affinity site on the cytosolic side of the membrane. This substrate binding site is subsequently reoriented to the extracellular face of the membrane where the binding affinity is decreased so that the substrate may be released. The final step of the transport cycle is the return of the binding site to its initial high affinity state (28). For MRP1 (and other ABC transporters), these steps

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2 The abbreviations used are: MRP1, multidrug resistance protein 1; ABC, ATP binding cassette; GSH, reduced glutathione; S-mGSH, S-methyl glutathione; DTT, dithiothreitol; LTC4, leukotriene C4; E217-βG, estradiol glucuronide; AMPPPN, adenylylimidodiphosphate; ATPγS, adenosine thiotriphosphate; Vγ1, orthovanadate ion; mAb, monoclonal antibody; BAEE, N-acetylbenzoyl-L-arginine ethyl ester; NBD, nucleotide binding domain.
of the transport cycle are coupled to the binding and/or hydrolysis of ATP, but the precise details of this coupling are not known. Nor is it known when and how GSH participates in this process. Because conventional vesicular transport assays do not readily allow the individual constituent steps of the transport cycle to be examined, we have developed a radioligand binding assay to investigate directly the binding of estrone sulfate to MRP1, to measure the effect of GSH on the binding of this substrate, and to determine how the binding of estrone sulfate and GSH is affected by nucleotide binding and hydrolysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—[6,7,2-3H]Estrone sulfate (57.3 Ci/mmol) and [14,15,19,20,3H]LTC₄ were purchased from PerkinElmer Life Sciences. LTC₄ was from Calbiochem (La Jolla, CA). Diphenylcarbamyl chloride-treated trypsin was from Roche Diagnostics (Laval, Quebec, Canada). mAbs MRPm6 and MRPr1 were a kind gift from Drs. R. J. Scheper and G. L. Scheffer (Amsterdam, Netherlands). Complete, EDTA-free protease inhibitor mixture was from Roche Diagnostics (Laval, Quebec, Canada). GSH, S-mGSH, ATP, AMPNP, ATPγS, sodium orthovanadate, E₆/₇BG, estrone 3-sulfate, DTT, and BAEE were from Sigma.

**Cell Culture and Membrane Preparation**—The H69 small cell lung cancer cell line and its doxorubicin-selected, MRP1-overexpressing H69AR-derivative cell line were cultured as described previously (29). To prepare plasma membranes, cells (~5 x 10⁶) were harvested, resuspended in buffer 1 (50 mM Tris, pH 7.4, 250 mM sucrose) containing 250 μM CaCl₂, and protease inhibitors, and subjected to argon cavitation (250 p.s.i., 5 min, 4 °C). Unexploded cells and debris were removed by low speed centrifugation (750 x g, 10 min, 4 °C), and the supernatant was loaded onto a 35% sucrose cushion. Following centrifugation (100,000 x g, 1 h, 4 °C), plasma membranes were harvested from the sucrose interface, diluted with buffer 2 (50 mM Tris, pH 7.4, 25 mM sucrose), and centrifuged again. The membrane pellet was resuspended in buffer 1 at ~10 mg of protein per ml and stored at ~70 °C for up to 3 months. Protein concentrations were measured using a Bio-Rad Bradford assay, with bovine serum albumin as a standard.

**[3H]Estrone Sulfate Binding Assays**—Membrane protein (10 μg) was allowed to equilibrate with [3H]estrone sulfate in the presence or absence of organic anion substrates and/or nucleotides in a total volume of 50 μl of hypotonic buffer 3 (50 mM HEPES, pH 7.4) at 23 °C for 1 h. The use of hypotonic buffer ensures that the membrane vesicles are burst open and, thus, that the MRP1 protein is accessible from both sides of the membrane. Following equilibration, 1.5 ml of ice-cold buffer 4 (20 mM HEPES, pH 7.4, 20 mM MgCl₂) was added, and samples were filtered immediately through a PerkinElmer Life Sciences unfilter GF/B plate using a Packard Filtermate Harvester, and washed twice with buffer 4. Tritium bound to the filter was determined using a PerkinElmer Life Sciences Top Count NXT Microplate Scintillation counter.

**Displacement/Stimulation Assays**—Binding assays were used to measure the displacement of estrone sulfate binding by other substrates of MRP1 or the increase in estrone sulfate binding in the presence of GSH analogues. Membrane proteins (10 μg) were incubated with [3H]estrone sulfate (35–40 nM) in the presence of (a) 3 mM S-mGSH and various concentrations of LTC₄ (3 mM to 10 μM), E₆/₇BG (300 nM to 3 mM), or methotrexate (1 mM to 3 mM) or (b) various concentrations of GSH or S-mGSH (1 mM to 10 mM), before filtering as described above. DTT (10 mM) was present in all experiments containing GSH to prevent oxidation to oxidized glutathione. Data were fitted with a sigmoidal dose-response curve by non-linear regression analysis (Equation 1).

\[
B = B_{\text{min}} + (B_{\text{max}} - B_{\text{min}}) / (1 + 10^{(\log EC_{50} - \log S)})
\]

(Eq. 1)

where \(B\) is the amount of estrone sulfate bound, \(B_{\text{max}}\) is maximal binding at this concentration of estrone sulfate, \(B_{\text{min}}\) is nonspecific binding, \([S]\) = concentration of substrate, and \(EC_{50}\) is the substrate concentration at half-maximal estrone sulfate binding.

**Saturation Binding Isotherms**—To determine parameters of maximal binding capacity (\(B_{\text{max}}\)) and binding dissociation constant (\(K_d\)), 10 μg of membrane protein was incubated with various concentrations of [3H]estrone sulfate (10 nM to 30 μM). This assay was done in the presence of 0.3, 1, 3, or 10 mM S-mGSH or GSH (plus 10 mM DTT) in the presence or absence of 1 mM E₆/₇BG. Nonspecific binding (in the presence of 1 mM E₆/₇BG) was subtracted from total binding to determine specific binding, and specific data were fitted with a one-site binding hyperbola (Equation 2).

\[
B = B_{\text{max}} \cdot [L] / (K_d + [L])
\]

(Eq. 2)

where \(B\) is the amount of estrone sulfate bound, \(B_{\text{max}}\) is maximal binding capacity for estrone sulfate, \([L]\) is the concentration of estrone sulfate, and \(K_d\) is the binding dissociation constant. The commercially available radioligand could not be used to generate [3H]estrone sulfate concentrations above 3 μM, and this was therefore achieved by supplementing with unlabeled estrone sulfate as previously described (30).

**Association Kinetics**—The rate of association of estrone sulfate with MRP1 in the presence of S-mGSH was determined by using the following three protocols. Comparison of the data from each protocol was used to ascertain the order of binding of GSH and estrone sulfate to MRP1. Firstly, membrane proteins (10 μg) were preincubated with 3 mM S-mGSH for 30 min in the presence or absence of 1 mM E₆/₇BG (for determination of nonspecific estrone sulfate binding). [3H]Estrone sulfate (40 nM) was added at selected time points (0–60 min) and then stopped by addition of buffer 4 and filtration as described above. Secondly, [3H]estrone sulfate (40 nM) and S-mGSH (3 mM) were mixed, with or without E₆/₇BG (1 mM), and membrane proteins (10 μg) were added at selected times (0–60 min) before filtering. Lastly, membrane proteins (10 μg) were preincubated with [3H]estrone sulfate (40 nM) for 30 min, with or without 1 mM E₆/₇BG, and S-mGSH (3 mM) was added at selected times (0–60 min) before filtering. For each of the above three protocols, specific estrone sulfate binding was calculated by subtracting nonspecific binding (in the presence of 1 mM E₆/₇BG) from total binding (in the absence of E₆/₇BG), for each time point. An exponential association curve (Equation 3) was fitted to the specific binding data by non-linear regression,

\[
B = B_{\text{max}}(1 - e^{-k_{\text{obs}}t})
\]

(Eq. 3)

where \(B\) is the estrone sulfate bound, \(B_{\text{max}}\) is maximal binding at this concentration of estrone sulfate, \(t\) is time, and \(k_{\text{obs}}\) is the observed rate constant.

**Addition of Nucleotides**—AMPNP or ATPγS was added to membrane proteins to a final concentration of 4 mM in the presence of 5 mM MgSO₄. Vanadate trapping of nucleotide was achieved by incubating membrane proteins at 37 °C for 30 min with 4 mM ATP, 5 mM MgSO₄, and 1 mM sodium orthovanadate (prepared as a 100 mM stock solution, pH 10, and boiled prior to use (31)). Excess ATP was removed by centrifugation (25,000 x g, 15 min, 4 °C), and the membranes were resuspended in buffer 3 containing 1 mM orthovanadate, ready for use in the estrone sulfate binding assays. Membrane proteins were subjected to the same conditions but without ATP as a control and were included in each assay to ensure there was no spurious effect of the nucleotide-trapping conditions.
GSH-stimulated Estrone Sulfate Binding by MRP1

[3H]LTC4 Transport to Measure Vanadate-induced Nucleotide Trapping Efficiency—Vanadate-induced nucleotide trapping by MRP1 was carried out as described above, except buffer 1 (sucrose buffer) was used in place of buffer 3 (hypotonic buffer), and following centrifugation to remove unbound ATP, the vesicles were resuspended in buffer 1 without vanadate and immediately used in LTC4 transport assays. As before, a sample subjected to the same conditions but without ATP was prepared as a control. [3H]LTC4 transport was carried out as described previously (5, 32). Briefly, 50 nM [3H]LTC4 (20 nCi per point), 10 mM MgCl2, and 4 mM ATP plus an ATP-regenerating system consisting of creatine kinase and creatine phosphate or 4 mM AMP were incubated with 2 μg of membrane protein at 23 °C for 1 min. The reaction was stopped by dilution in ice-cold buffer 1 and rapidly filtered as described for the binding assays. Uptake in the presence of AMP was subtracted from uptake in the presence of ATP to determine ATP-dependent transport. The residual ATP-dependent [3H]LTC4 uptake in the vanadate-trapped sample was compared with the control sample as a measure of vanadate-induced nucleotide trapping efficiency.

Limited Trypsin Digestion of MRP1—Membranes (0.25 mg of protein ml−1) in buffer 3 were incubated alone, or with 10 mM DTT, 10 mM estrone sulfate, 10 mM GSH (plus 10 mM DTT), 10 mM S-mGSH or various combinations of these reagents, for 30 min on ice. Diphencylcarbamyl chloride-treated trypsin was then added at trypsin:protein ratios of 1:5000 to 2.5:1 (w/w) for 15 min at 37 °C. Reactions were stopped by addition of Laemmli sample buffer containing leupeptin (16.7 μg ml−1) and phenylmethylsulfonyl fluoride (10 μM). Samples (2 μg of protein) were resolved on a 7% acrylamide gel and immunoblotted. Tryptic fragments of MRP1 were detected by chemiluminescence using the primary mAbs MRPM6 (1:1000) and MRPr1 (1:5000), whose epitopes lie at the connecting the first and second membrane spanning domains (amino acids 238–247), respectively (33).

The direct effect of estrone sulfate, GSH, and/or DTT on trypsin activity per se was assayed using the model substrate BAEE. Diphencylcarbamyl chloride-treated trypsin (500 units ml−1 in 1 mM HCl) was incubated alone, or in the presence of 50 μM estrone sulfate, 10 mM DTT, 10 mM GSH, 10 mM S-mGSH or combinations of these reagents at 23 °C for 30 min. The trypsin (200 μl) was then added to 0.25 mM BAEE (3 ml), and trypsin activity was determined as the rate of increase of absorbance at 250 nm over a 10 min period.3

Data Analysis—All non-linear regression analyses were carried out using GraphPad Prism 3.0 (San Diego, CA). Data sets contain a minimum of three independent experiments, and data are depicted as means ± S.E. Statistical comparisons were carried out using one-way analysis of variance with a Tukey post-hoc test. Differences were considered statistically significant when p was <0.05.

RESULTS

Requirement of GSH for Specific Estrone Sulfate Binding—Radioligand binding assays were developed to study the interaction of estrone sulfate and GSH with MRP1. Approximately 250,000 dpm of [3H]estrone sulfate was added to a reaction (10 μg of membrane), of this <1% was bound by the filters. As shown in Fig. 1A, the level of [3H]estrone sulfate binding to H69AR (MRP1+) membranes in the absence of GSH was ~1,100 dpm, the same as that to membranes from parental H69 (MRP1+) cells, indicating low level nonspecific binding to non-MRP1 components of the membrane preparation. Consistent with this conclusion, the amount of estrone sulfate binding could not be displaced by the MRP1 substrate E217βG. The inability to measure specific estrone sulfate binding could also not be overcome by increasing concentrations of [3H]estrone sulfate, at least up to 10 μM (data not shown). In contrast, the addition of GSH produced a substantial increase in [3H]estrone sulfate binding to the MRP1+ membranes while having no effect on the MRP1− membranes (Fig. 1A), indicating that specific binding of estrone sulfate to MRP1 occurs in the presence of GSH. The specificity of this GSH-induced binding was confirmed by the ability of excess E217βG to reduce [3H]estrone sulfate binding to the nonspecific level seen in the absence of GSH while having no effect on the MRP1− membranes. The specific binding of estrone sulfate to MRP1 could also be readily measured in the presence of the non-reducing GSH analogue S-mGSH. Together these data indicate that membranes from H69AR cells display a specific binding site for estrone sulfate but only in the presence of GSH (or S-mGSH). The lack of binding to H69 membranes indicates that the binding to membranes from resistant cells was to the MRP1 transporter. Support for the specific binding component as MRP1 was provided through heterologous displacement assays using several other known substrates of MRP1. Thus, LTC4 (IC50 0.95 μM), E217βG (IC50 90 μM), and methotrexate (IC50 1.8 μM) all produced concentration-dependent reductions in estrone sulfate binding to levels observed in the H69 (MRP1−) membranes (Fig. 1B). The order of potency to displace estrone sulfate binding matched the relative uptake affinities for MRP1 displayed by these substrates (5, 6, 20).

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Effect of GSH or S-mGSH on the affinity and binding capacity of MRP1 for [3H]estrone sulfate

Maximal binding capacity ($B_{\text{max}}$) and binding dissociation constants ($K_d$) from binding isotherms for estrone sulfate (0–5 μM) to MRP1 (10 μg membrane protein) were determined in the presence of the indicated concentrations of S-mGSH or GSH (plus 10 mM DTT). Data are means ± S.E. (n = 3).

| Concentration (mM) | $K_d$ (μM) | $B_{\text{max}}$ (pmol mg$^{-1}$) |
|-------------------|-----------|-------------------------------|
| S-mGSH 0.3        | 0.54 ± 0.13 | 37 ± 7                        |
|                   | 1         | 0.69 ± 0.12                   | 67 ± 4                        |
|                   | 3         | 0.59 ± 0.05                   | 87 ± 7                        |
|                   | 10        | 0.65 ± 0.15                   | 123 ± 16                      |
| GSH 0.3           | 1.71 ± 0.16 | 41 ± 9                        |
|                   | 1         | 1.81 ± 0.16                   | 65 ± 6                        |
|                   | 3         | 1.48 ± 0.06                   | 83 ± 8                        |
|                   | 10        | 1.68 ± 0.17                   | 111 ± 11                      |

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TABLE 2
Effect of order of ligand addition on association parameters for GSH-enhanced [3H]estrone sulfate binding to MRP1

| Assay condition | Association parameters<sup><em>b</em></sup> |<sup><em>c</em></sup> |
|-----------------|---------------------------------------------|
| k<sub>obs</sub> | min<sup>−1</sup> |<sup><em>a</em></sup> | t<sub>1/2</sub> | min<sup>−1</sup> |<sup><em>a</em></sup> |
| 1 | 0.355 ± 0.027 | 2.00 ± 0.18 |
| 2 | 0.167 ± 0.010<sup>c</sup> | 4.19 ± 0.27<sup>c</sup> |
| 3 | 0.173 ± 0.011<sup>c</sup> | 4.05 ± 0.26<sup>c</sup> |

<sup><em>a</em></sup> Assay conditions were as follows: 1) membranes were preincubated with 3 mM S-mGSH before adding [3H]estrone sulfate; 2) S-mGSH and [3H]estrone sulfate were added to membranes at the same time; and 3) membranes were preincubated with [3H]estrone sulfate before adding 3 mM GSH.

<sup><em>b</em></sup> Observed association rate constants (k<sub>obs</sub>) and time taken for estrone sulfate binding to reach half the equilibrium binding level (t<sub>1/2</sub>) were determined for [3H]estrone sulfate (40 nM) binding to MRP1 (10 μg of membrane protein) (Fig. 4). Data are means ± S.E. from at least three independent experiments.

<sup><em>c</em></sup> Denotes a significant difference from assay condition 1 (p < 0.001).

FIGURE 5. Tryptic digestion profiles of MRP1 in the presence and absence of GSH. A, H69AR (MRP1<sup>−</sup>) membrane protein (0.25 mg ml<sup>−1</sup>) was preincubated alone or with 10 mM GSH and 10 mM DTT for 30 min on ice, before addition of trypsin at trypsin:protein ratios of 2.5:1 to 1:5000 (w/w). Samples (2 μg of protein) were resolved on a 7% acrylamide gel and immunoblotted with mAb MRPm6 (1:1000, 5-min film exposure). Arrows mark the position of the intact MRP1, the larger COOH-proximal C1 fragment, and the smaller COOH-terminal C2 fragment of MRP1. B, shown is a shorter exposure (30 s) of the first lane shown in A to illustrate the relatively low level of tryptic fragments present at low trypsin concentrations.

FIGURE 6. Effect of nucleotide binding on [3H]estrone sulfate binding to MRP1. A, binding of [3H]estrone sulfate (1 μM) to H69 (MRP1<sup>−</sup>) (white bars) or H69AR (MRP1<sup>−</sup>) (black bars) membranes (10 μg of protein) was measured in the presence or absence of 3 mM GSH (plus 10 mM DTT) and/or 4 mM AMPPNP, or 4 mM ATP-S. Bars are means ± S.D. from a representative experiment carried out in triplicate. B, specific binding of [3H]estrone sulfate (0.01–10 μM) to MRP1 (10 μg of membrane protein) was measured in the presence of 3 mM S-mGSH, in the absence of nucleotide (closed circles) and in the presence of 4 mM AMPPNP (open circles). Data are normalized to the B<sub>max</sub> and points are means ± S.E. from at least three independent experiments.

GSH Binding Causes a Conformational Change in MRP1—The mechanism by which GSH binding allows estrone sulfate to bind MRP1 is not clear. One possibility is that GSH binding causes a conformational change in MRP1 that opens up a binding site for estrone sulfate. Altered sensitivity to proteolysis has been previously reported to reflect conformational changes in several different membrane proteins (36–39). Consequently, limited trypsin digestion of MRP1 was carried out in the presence and absence of GSH and estrone sulfate. As shown in Fig. 5A, digestion of MRP1 with increasing concentrations of trypsin produces first a larger 80-kDa COOH-proximal fragment (C1) and then the smaller 36- to 40-kDa COOH-terminal fragment (C2), as detected using mAb MRPm6 (COOH-terminal epitope) and characterized previously (19, 40).

In the presence of 50 μM estrone sulfate, there was no change in this digestion profile (data not shown). In the presence of 10 mM GSH (and DTT), however, a different profile was observed (Fig. 5A). Thus, the intact MRP1 protein was more susceptible to tryptic digestion than the control, yet there was also a protective effect against cleavage of the C1 fragment to produce the C2 fragment. This protection of the COOH terminus of MRP1 afforded by GSH agrees with a previously published report (41). A similar effect on the digestion profile was observed when S-mGSH was substituted for GSH (data not shown). Furthermore, the presence of estrone sulfate in addition to GSH did not significantly change the profile compared with GSH alone (data not shown). In contrast to the ability of GSH to protect the COOH terminus of MRP1, GSH had no effect on the trypsin-mediated cleavage of more NH<sub>2</sub>-proximal regions of MRP1 as detected with mAb MRPm1 (epitope in intracellular loop 3 between the first and second membrane-spanning domains) (data not shown).

The relatively long exposure time (5 min) used for the immunoblot shown in Fig. 5A, gives the appearance of large scale breakdown of MRP1 to the C1 fragment even at very low concentrations of trypsin. However, as seen in Fig. 5B, the short exposure time (30 s) of lane 1 shows this is simply an artifact produced by the overexposure of the higher molecular weight bands to detect the C2 COOH-terminal fragments. The possibility that the effect of GSH (plus DTT) or S-mGSH on the susceptibility of MRP1 to tryptic digestion was due to a direct effect on the activity of trypsin was excluded using a BAAE assay (data not shown). Our results suggest that binding of GSH to MRP1 induces a conformational change that appears specific to the COOH-terminal half of the protein, which subsequently enhances estrone sulfate binding.

Effect of ATP Binding on Estrone Sulfate Binding—The studies above describe estrone sulfate and GSH binding to MRP1 in the nucleotide-free state. To more fully understand the role of GSH in estrone sulfate transport, studies in the presence of nucleotides were undertaken. Thus, binding of estrone sulfate to MRP1 was measured in the presence of AMPPNP (4 mM), a non-hydrolyzable analogue of ATP, at a concentra-
tion we showed previously was sufficient to bind to both NBDs of MRP1 (42). As observed with nucleotide-free MRP1 in the absence of GSH, there was only low level nonspecific binding of estrone sulfate, but in the presence of GSH, MRP1-specific binding was readily detectable (Fig. 6A). However, the level of GSH-enhanced MRP1-specific estrone sulfate binding in the presence of AMPPNP was significantly reduced compared with binding in the absence of nucleotide. Comparable results were obtained when 4 mM ATP (a very slowly hydrolyzable ATP analogue) was used instead of AMPPNP.

Further examination of estrone sulfate binding over a full concentration range in the presence of 3 mM S-mGSH showed that AMPPNP binding to MRP1 caused a substantial shift in the binding curve (Fig. 6B). This represents a significant decrease in the affinity for estrone sulfate when AMPPNP binds (Kd 2.52 ± 0.12 μM) compared with the nucleotide-free state (Kd 0.59 ± 0.05 μM). However, no change in the Bmax for estrone sulfate was observed. Similar results were obtained when GSH was used instead of S-mGSH, i.e. a significant increase in Kd of 2.52 ± 0.12 μM, with no change in Bmax. These data are summarized in Table 3.

Effect of ATP Hydrolysis on Estrone Sulfate Binding—Having observed that binding of nucleotide caused a switch from high to low affinity for estrone sulfate binding, the next step was to examine the effect of ATP hydrolysis. The immediately post-hydrolysis state of MRP1 (ADP-P) can be mimicked by vanadate (V4-) induced trapping of ADP, where V4 replaces P, to form the stable intermediate ADP-V4 (43). The efficiency of ADP-V4 trapping under the conditions used in this study was assayed by measuring the residual MRP1-mediated LTC4 transport, and these data indicated that 88 ± 2% of the MRP1 was in the ADP-V4-trapped state. As observed in the nucleotide-free and AMP-PNP-bound states, GSH or S-mGSH was required for specific binding of estrone sulfate to MRP1 in the ADP-V4-trapped state (data not shown). The effect of ADP-V4 trapping on the binding of estrone sulfate in the presence of 3 mM S-mGSH is shown in Fig. 7. Thus, the Kd for estrone sulfate in the ADP-V4-trapped state remained low affinity (Kd 3.31 ± 0.31 μM) compared with the nucleotide-free conformation (Kd 0.59 ± 0.05 μM) and similar to the AMP-PNP-bound state. The Bmax for estrone sulfate (Table 3) also appeared to decrease moderately in the ADP-V4-trapped state (Bmax 87 ± 7 pmol/mg) compared with the nucleotide-free state (Bmax 144 ± 67 pmol/mg), but this difference was not statistically significant (p > 0.1). Similar results were observed during transition through the catalytic cycle when GSH was used rather than S-mGSH, and these results are summarized in Table 3.

Effect of ATP Binding and Hydrolysis on GSH Binding—The above experiments established that GSH or S-mGSH is required for binding of estrone sulfate to MRP1 in both the nucleotide-bound and the post-ATP hydrolysis states of the transporter. It was therefore of interest to know whether nucleotide binding and/or hydrolysis affects GSH binding itself. As detailed earlier, it is technically not possible to determine the affinity of MRP1 for GSH or S-mGSH directly. Consequently, the potency of S-mGSH to increase estrone sulfate binding was used as an indirect measure of the affinity of MRP1 for this GSH analogue. As shown in Fig. 8A, the level of S-mGSH-stimulated estrone sulfate binding was lower in the presence of AMPPNP (3500 dpm) than in the nucleotide-free state (7300 dpm). This is due to the decreased affinity for estrone sulfate when AMPPNP is bound. However, the EC50 of S-mGSH in the presence of AMPPNP was 0.80 ± 0.18 mM, which is comparable to the EC50 in the nucleotide-free state (0.77 ± 0.06 mM), suggesting the affinity for S-mGSH is not altered by nucleotide binding.

![FIGURE 7. Effect of ATP hydrolysis on [3H]estrone sulfate binding to MRP1. Specific binding of [3H]estrone sulfate (0.01–30 μM) to MRP1 (10 μg of membrane protein) was determined in the presence of 3 mM S-mGSH (closed circles) and in the ADP-V4-trapped state (open circles). Data are normalized to the Bmax and points are means ± S.E. from at least three independent experiments.](image)

![FIGURE 8. S-mGSH concentration-dependent binding of [3H]estrone sulfate to MRP1, the effect of nucleotide binding or hydrolysis. Binding of [3H]estrone sulfate (40 nM) to MRP1 (10 μg of membrane protein) was determined in the presence of increasing concentrations (1 μM to 10 mM) of S-mGSH. A, the nucleotide-free state (closed circles) and AMPPNP-bound state (open circles); B, the nucleotide-free (closed circles) and ADP-V4-trapped state (open triangles). Data are normalized to the Bmax and points are means ± S.E. from at least four independent experiments.](image)
estrone sulfate (Fig. 9, nal region of the protein) that opens up a high affinity binding site for MRP1 causes a conformational change in MRP1 (in the COOH-terminal region of MRP1 was induced by both GSH and its non-reducing analogue 44). The same change in the proteolytic sensitivity of the COOH-terminus of MRP1 caused estrone sulfate alone to react as if it were a substrate, but not as if it were a ligand. In contrast, in the absence of GSH, estrone sulfate in the absence of GSH, although this was only significant at relatively high estrone sulfate concentrations (>20 μM). Therefore, it seems that MRP1 is capable of binding estrone sulfate in the absence of GSH but not at low affinity.

In the absence of GSH no specific binding of estrone sulfate to MRP1 was detected (Fig. 1); however, we have previously reported that MRP1-mediated uptake of estrone sulfate into inside-out membrane vesicles could occur in the absence of GSH, albeit at a very low level (8, 34). Whereas others have been unable to detect this (17), we also showed previously that estrone sulfate could inhibit LTC4 binding and transport in the absence of GSH, although this was only significant at relatively high estrone sulfate concentrations (>20 μM). Therefore, it seems that MRP1 is capable of binding estrone sulfate in the absence of GSH but with such a low affinity that it is beyond the detection level of the radioligand binding assay described here.

Our present findings also indicate that the low level binding of estrone sulfate by MRP1 in the absence of GSH has no detectable effect on the conformation of the transporter, because estrone sulfate alone did not affect its tryptic digestion profile. In contrast, GSH binding does induce a conformational change in MRP1, as suggested previously (41, 44). The same change in the proteolytic sensitivity of the COOH-terminal region of MRP1 was induced by both GSH and its non-reducing analogue S-mGSH (Figs. 5 and 9, step II). We have also demonstrated before that both of these analogues are capable of stimulating estrone sulfate transport and, therefore, that the sulfhydryl group of GSH is not required (8). In fact the cysteine residue of GSH can be replaced with several other non-polar amino acids and still stimulate estrone sulfate transport, in some cases even better than GSH (34). The present ligand binding assays showed that the apparent affinity of MRP1 for both GSH and S-mGSH is the same (Fig. 2), as was previously suggested by transport studies (8). The notably different abilities of GSH and S-mGSH to stimulate estrone sulfate transport and binding are instead due to a difference in the affinity for estrone sulfate itself. Thus, estrone sulfate binds to MRP1 with a significantly higher affinity in the presence of S-mGSH than GSH (Fig. 3 and Table 1). This could be because of a direct interaction between GSH and estrone sulfate in a common sub-strate binding pocket of MRP1 which is much stronger with the presence of the additional methyl group of S-mGSH, perhaps due to its larger size or hydrophobicity. Alternatively, GSH/S-mGSH may bind to a site distinct from that where estrone sulfate binds, and the conformational changes induced in MRP1 by S-mGSH and GSH may not be exactly the same. Although involving essentially the same regions of the protein (and hence not affecting trypsin sensitivity), the increased size and/or hydrophobicity of S-mGSH may cause a slightly larger movement or a variation in side-chain orientation that results in a higher affinity binding site for estrone sulfate. The fact that GSH or S-mGSH was required for measurement of estrone sulfate binding at each stage of the catalytic cycle tested, yet there is no evidence that GSH is co-transported with estrone sulfate, would argue against a direct interaction between the two ligands. For this reason, we favor the second explanation.

In the presence of the non-hydrolyzable AMPPNP, the affinity for estrone sulfate decreased significantly (Table 3). This observation may be explained by an ATP-dependent reorientation of the estrone sulfate binding site to its low affinity state, which is presumably accessible to the extracellular face of the membrane to facilitate release of estrone sulfate (Fig. 9, steps IV–VII). It has previously been reported that AMPPNP binding to MRP1 causes a substantial conformational change in the protein (44), as would be required for this substrate reorientation. We have also observed a conformational change in MRP1 induced by AMPPNP as detected by trypsin sensitivity assays. On the other hand, we recently reported that AMPPNP binding has no effect on the affinity of MRP1 for LTC4 (42). The reasons for these apparently different responses to AMPPNP are not clear. In the previous study membranes from an insect cell expression system were used, in contrast to the human lung cancer cell membranes used here, and potentially the very different membrane composition of the two cell types may affect MRP1 and its interaction with AMPPNP. Alternatively, it may be that the changes induced by AMPPNP binding do not affect binding of all MRP1 substrates in the same way. However, for both LTC4 and estrone sulfate, the slowly hydrolyzable ATPγS reduced substrate binding, and, consequently, the evidence supports the conclusion that ATP binding is sufficient for reconfiguration of the substrate binding site.

4 A. Rothnie and S. P. C. Cole, unpublished observations.
In our model shown in Fig. 9, we have suggested that the reorientation of the estrone sulfate binding site occurs only when ATP binds to both NBDs. The reasons for this are severalfold: (i) at the concentration used in this study, AMPPNP binds to both NBDs (42); (ii) it has been reported that ATP binding to NBD1 promotes the subsequent binding of ATP to NBD2 (45); (iii) mutations in NBD2 of MRP1, which increase the affinity for ATP, potentiates the reorientation of the Ltc_2 binding site to low affinity (46); and (iv) the related ABCC protein, the cystic fibrosis transmembrane conductance regulator (ABCC7) like MRP1 has non-equivalent NBDs, and ATP binding at both NBDs of cystic fibrosis transmembrane conductance regulator is required for opening of this chloride channel (47, 48). Thus, when both cystic fibrosis transmembrane conductance regulator NBDs bind ATP, a tight dimerization of the NBDs occurs, which leads to channel opening (49), and it has been proposed that such a tight dimerization of the NBDs may be a common feature of the translocation mechanism for all ABC proteins.

Unlike estrone sulfate, the apparent affinity of MRP1 for GSH/S-mGSH was not altered by the binding of AMPPNP (Fig. 8A). This suggests that, although the estrone sulfate binding site was reoriented to a low affinity state, the GSH binding site was not significantly altered (Fig. 9, steps VI–VII). As previously mentioned, this supports the idea that GSH does not simply play a "space-filling" role in a common substrate binding pocket alongside estrone sulfate. Instead it seems more likely that GSH affects estrone sulfate binding by an allosteric mechanism. In addition, because MRP1 can also transport GSH alone or together with substrates such as vincristine (14), and assuming that nucleotides play a common mechanistic role in the transport of different substrates, then these findings could be construed to indicate the presence of two distinct GSH binding sites: one where GSH undergoes transport, and a second where GSH elicits allosteric effects on MRP1 without being translocated across the membrane. Such an idea has been proposed previously on the basis of mutations experiments where the mutation of a single residue prevents GSH transport, yet GSH-stimulated estrone sulfate transport is only mildly affected (50, 51), and vice versa (52), but this remains to be directly proven.

Following hydrolysis of ATP, as mimicked experimentally by vana-date-induced trapping of ADP by MRP1, the estrone sulfate binding site on the transporter remained in a low affinity state (Fig. 7). However, at this stage of the catalytic cycle, unlike ATP binding, the apparent affinity of MRP1 for GSH/S-mGSH was also decreased (Fig. 8B). Because we can find no evidence that GSH is co-transported with estrone sulfate, it is quite conceivable that the GSH is released back on the cytoplasmic side of the membrane (Fig. 9, step VIII). However, even though estrone sulfate does not cause an associated increase in GSH transport in the manner that vincristine does (14), there is the formal possibility that the "basal" level of GSH transport (i.e. the rate of transport of GSH in the absence of other substrates) might be sufficient to support co-transport of estrone sulfate (8). However, the potency with which GSH mediates its effects on estrone sulfate binding and transport (EC50 0.5–0.8 mM) is substantially higher than the estimated affinity for GSH undergoing basal transport (Km ~ 4 mM (35)), suggesting that these are two quite different processes. In addition, release of GSH on the cytoplasmic side of the membrane as we suggest would further reduce the affinity for estrone sulfate at the external face, thus accelerating the dissociation of estrone sulfate and elevating the rate of transport.

Clearly the model presented in Fig. 9 is not complete, because the steps that result in the restoration of high affinity estrone sulfate binding after transport are not described. Thus far the only information available on this aspect of the MRP1 transport cycle has been provided by Yang et al. (53). In their study they showed that nucleotide dissociation from NBD1 is required for resetting of MRP1 and allowing a new cycle of transport to commence. However, it is not known if this is simply the release of the bound ATP, or whether the ATP must first be hydrolyzed, and it is the subsequent release of ADP that is required.

How the mechanism proposed here for GSH-stimulated transport of estrone sulfate relates to the GSH-dependent transport of other MRP1 substrates remains to be tested. However, it seems reasonable to postulate that substrates, such as the nicotine metabolite 4-(methylisotrosmosino)-1-(3-pyridyl)-1-butanol-O-glucuronide (16) and glutathionylquinolineoxide (17), may utilize a similar mechanism. Like estrone sulfate, ATP-dependent vesicular transport of these molecules is undetectable in the absence of GSH. GSH stimulates their transport, yet these metabolites either inhibit or have no effect on the basal level of GSH transport by MRP1. It also remains to be determined how the mechanism of vincristine and GSH co-transport, and drug-stimulated GSH efflux, compare with the mechanism proposed here.

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