Dynamics of Glutathione Conjugation and Conjugate Efflux in Detoxification of the Carcinogen, 4-Nitroquinoline 1-Oxide: Contributions of Glutathione, Glutathione *S*-Transferase, and MRP1[†]

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ABSTRACT: 4-Nitroquinoline 1-oxide (NQO) is a reactive electrophile with potent cytotoxic as well as genotoxic activities. NQO forms a conjugate, QO-SG, with glutathione, which greatly reduces its chemical reactivity. Previous studies demonstrated that glutathione *S*-transferase (GST) P1a-1a and multidrug resistance protein (MRP) 1/2 act in synergy to confer resistance to both cyto- and genotoxicities of NQO, whereas protection afforded by GSTP1a-1a or MRP alone was much less. To better understand the role of glutathione, GSTP1a-1a, and MRP1 in NQO detoxification, we have characterized the kinetics and cofactor requirements of MRP1-mediated transport of QO-SG and NQO. Additionally, using recombinant GSTP1a-1a and physiological conditions, we have examined the enzymatic and nonenzymatic formation of QO-SG. Results show that MRP1 supports efficient transport of QO-SG with a K_m of 9.5 μM and a V_{max} comparable to other good MRP1 substrates. Glutathione or its *S*-methyl analogue enhanced the rate of ^{3}H-QO-SG transport, whereas QO-SG inhibited the rate of ^{3}H-glutathione transport. These data favor a mechanism for glutathione-enhanced, MRP1-mediated QO-SG transport that does not involve cotransport of glutathione. NQO was not transported by MRP1 either alone or in the presence of *S*-methyl glutathione. Transport of ^{3}H-NQO was observed in the presence of glutathione, but uptake into MRP1-containing vesicles was entirely attributable to its conjugate, QO-SG, formed nonenzymatically. While the nonenzymatic rate was readily measurable, enzyme catalysis was overwhelmingly dominant in the presence of GSTP1a-1a (rate enhancement factor, $(k_{cat}/K_m)/k_2$, $\sim 3 \times 10^6$). We conclude that MRP1 supports detoxification of NQO via efficient, glutathione-stimulated efflux of QO-SG. While nonenzymatic QO-SG formation and MRP1-mediated conjugate efflux result in low-level protection from cyto- and genotoxicities, this protection is greatly enhanced by coexpression of GSTP1-1 with MRP1. This result emphasizes the quantitative importance of enzyme-catalyzed conjugate formation, a crucial determinant of high-level, MRP-dependent protection of cells from NQO toxicity.

The detoxification of xeno- and endobiotic electrophiles, including genotoxic carcinogens and mutagens, frequently involves their conjugation with glutathione (GSH).[1] These conjugation reactions, catalyzed by glutathione *S*-transferases (GSTs), generally render the electrophiles less reactive and hence less toxic (*1, 2*). However, GSH conjugation of the lipophilic electrophiles also makes them more water soluble and impermeable to the plasma membrane. Consequently, mechanisms have evolved to remove these amphiphilic conjugates from the cell by ATP-dependent efflux transporters that include several members of the MRP family of membrane-associated transport proteins (*3*−*8*).

Previously, our laboratory and others have examined the detoxification of the model carcinogen, 4-nitroquinoline 1-oxide (NQO). Metabolites of NQO readily form nucleic acid adducts resulting in the carcinogenic activity observed in animal models (*9*−*11*). In addition, NQO has potent cytotoxic activity with IC_{50} in the nanomolar range (*12*). Although NQO is a good substrate for GSTP1-1 with relatively slow nonenzymatic rates of conjugation with GSH (*13*, *14*), expression of GSTP1a-1a alone conferred only modest protection from genotoxicity (DNA adduct formation) and no protection from cytotoxicity (*12*, *15*). However, coexpression of MRP1 (ABCC1) or MRP2 (ABCC2) with GSTP1a-1a revealed that MRP and GSTP1a-1a operate in synergy to confer high-level protection from both the cyto- and genotoxicities of NQO (*15*, *16*). These results indicated that removal of the GSH conjugate of NQO, QO-SG, in addition to its formation, might be essential for full detoxification. A surprising aspect of these studies was the finding that, despite the very slow rates of nonenzymatic formation of QO-SG reported, expression of MRP1 or MRP2 in the absence of GST conferred a low but measurable (1.5−4-fold) resistance to NQO toxicity (*15*, *16*).

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[1] Abbreviations: AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; DTT, dithiothreitol; GSH, glutathione; GST, glutathione *S*-transferase; GSTP1-1 and GSTP1a-1a, human GST P1 isozyme and its "a" allele; MRP, multidrug resistance (or resistance-associated) protein; NQO, 4-nitroquinoline 1-oxide; QO-SG, GSH conjugate of NQO, 4-(glutathione-*S*-yl)-quinoline 1-oxide.

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To explain these observations and in so doing better understand the roles of GSH, GSTP1a-1a, and MRP1 in NQO detoxification, the studies described herein were done. Utilizing cell-free systems, we have characterized the kinetics and cofactor requirement of MRP1-mediated transport of QO-SG and NQO. These studies suggest a mechanism for GSH-augmented transport of QO-SG that may apply generally to MRP1-mediated transport of GSH conjugates. In other experiments using recombinant purified GSTP1a-1a under physiological pH and temperature, we have examined the relative contributions of enzymatic versus nonenzymatic conjugation of NQO with GSH. The kinetic parameters described for the dynamics of NQO detoxification provide insights into the quantitative roles played by GSH, GSTP1a-1a, and MRP1 in protecting cells from this carcinogenic compound.

EXPERIMENTAL PROCEDURES

Materials. [Glycine-2-^{3}H]-GSH (52.0 Ci/mmol) and [6,7-^{3}H(N)]-estrone-3-sulfate (46.0 Ci/mmol) were purchased from Perkin−Elmer Life Sciences (Boston, MA). [3-^{3}H]-4-Nitroquinoine 1-oxide (20 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). NQO, GSH, *S*-methyl GSH, ATP, β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP), and acivicin were purchased from Sigma (St. Louis, MO). Creatine kinase and creatine phosphate were obtained from Roche Molecular Biochemicals (Indianapolis, IN).

Cell Lines and Culture. All cell lines were derived from parental MCF7/WT human breast carcinoma cells, which express negligible MRP1 and no GSTP1-1. Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 10 μg/mL ciprofloxacin at 37 °C, with 5% CO_2. The transgenic cell line overexpressing MRP1 (MCF7/MRP1−10) was generated by stable retroviral transduction with an MRP1-containing expression vector as described previously (*16*). MCF7/MRP1−10 cells were maintained in 0.5 mg/mL G418 selecting drug until just prior to vesicle preparation.

Synthesis and Analysis of QO-SG. The GSH conjugate of NQO was prepared in a 20 mL reaction containing 0.1 mM NQO, 1 mM GSH, 0.1 M potassium phosphate (pH 7.5), 50 μg/mL bovine serum albumin, and ∼0.2 μg/mL of recombinant GSTP1a-1a prepared as described previously (*17*). The reaction proceeded to completion at 25 °C and was acidified with perchloric acid to a final concentration of 10%. Samples were incubated at 0 °C for 10−15 min, and precipitate was removed by microfuge centrifugation. The supernatant was loaded onto a Waters (Milford, MA) Oasis HLB 3 cm^3 cartridge. The cartridge was rinsed with 5 mL of ddH_2O, and QO-SG was eluted in 3 mL of 30% methanol. The eluate was dried under nitrogen and redissolved in ddH_2O. The purity of QO-SG was verified by analytical HPLC (below) and by electrospray mass spectrometry (*15*). Conjugate concentration was determined by absorbance at 350 nm (ϵ = 15.3 mM^{-1} cm^{-1}) (*15*).

Radiolabeled QO-SG was prepared as described above with the following modifications. Reactions, 200 μL to 4 mL, contained 100 μM NQO and 200 μM total GSH including [glycine-2-^{3}H]-GSH to specific activities of 0.062 or 1.22 μCi/mmol. QO-SG was purified by HPLC using a Beckman C_{18} reverse-phase column and isocratic elution at 1 mL/min with a 9:1 mixture of 5% acetonitrile and 0.1% trifluoroacetic acid/methanol; QO-SG eluted at ∼31 min as described (*15*). Additional purification was accomplished using the Oasis HLB 3 cm^3 cartridge (Waters, Milford, MA) as described above.

ATP-Dependent, MRP1-Mediated Uptake into Inside-Out Vesicles. Membrane vesicles were prepared from MRP1-minus (MCF7/WT) or MRP1-positive (MCF7/1−10) cells as described (*16*, *18*). Transport studies were accomplished as described previously (*16*, *18*) but with the following modifications: a creatine kinase/creatine phosphate ATP regenerating system was included with the transport mixture, and a preincubation of vesicles with acivicin to minimize GSH catabolism by γ-glutamyl transpeptidase was added (*19*). Briefly, a total reaction volume of 50 μL containing 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 250 mM sucrose, 4 mM ATP or AMP-PCP (non-hydrolyzable ATP analog), 10 mM creatine phosphate, 100 μg/mL creatine kinase, and varying concentrations of radiolabeled substrates was warmed to 37 °C. The reaction was initiated by the addition of MRP1 expressing (MCF7/MRP1−10) or control (MCF7/WT) membrane vesicles (∼25−33 μg/50 μL reaction) that had been pretreated with 0.5 mM acivicin, for 10 min at 37 °C. A total of 10 μL aliquots were removed at specified times. For some transport experiments, unlabeled NQO, GSH, or *S*-methyl glutathione was added to the reaction mixture. Results reported as ATP-dependent uptake were calculated by subtracting transport observed in the presence of the AMP-PCP control from that observed in the presence of ATP. In transport studies utilizing GSH, complete reduction of the GSH thiol was assured by treating GSH stocks with 100 mM dithiothreitol (DTT) followed by 3 ethyl acetate extractions to remove DTT. Initial velocities were fitted to the Michaelis−Menten equation using Synergy KaleidaGraph 3.5 software for the Macintosh, and kinetic parameters were calculated.

Kinetics of Nonenzymatic and GSTP1a-1a-Catalyzed QO-SG Formation. The kinetics of enzyme catalysis of QO-SG formation was determined using a spectrophotometric assay adapted from Stanley and Benson (*20*). QO-SG formation was monitored at 37 °C using a Beckman DU 7400 spectrophotometer with a temperature controller. Reaction mixtures contained 0.1 M potassium phosphate (pH 7.5), 1 mM GSH, 50 μg/mL bovine serum albumin, ±24 ng/mL purified recombinant GSTP1a-1a, and 10−100 μM NQO. Reactions were initiated by the addition of NQO and initial velocities of QO-SG formation calculated from the change in absorbance at 350 nm ($\Delta\epsilon$ = 7.2 mM^{-1} cm^{-1}). Achievable substrate concentrations were limited by absorbance and aqueous solubility; hence, NQO concentrations were well below the K_m for GSTP1a-1a. Accordingly, data were fitted to the equation,

$$\frac{v}{E^T} = \frac{k_{cat}/K_m[\mathrm{NQO}]}{1 + \dfrac{[\mathrm{NQO}]}{K_m}}$$

which allowed an accurate estimation of k_{cat}/K_m, even though K_m and k_{cat} could not be determined separately [discussed by Kolm et al. (*21*)].

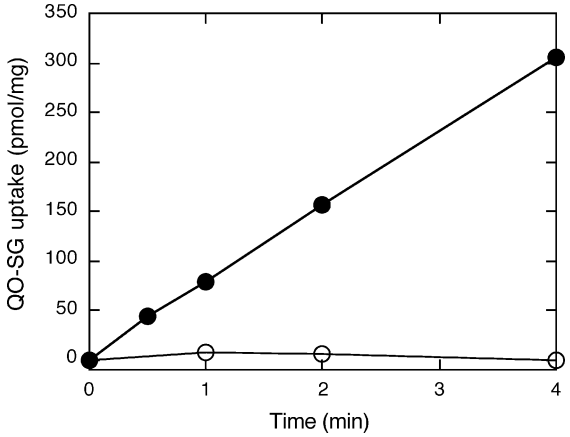

FIGURE 1: MRP1 mediates ATP-dependent QO-SG transport. Shown is ATP-dependent uptake of ^{3}H-QO-SG (6 μM) into inside-out membrane vesicles derived from MRP1-minus (MCF7/WT cells, ○) or MRP1-positive (MCF7/MRP1−10 cells, ●) cells from a typical experiment. Uptake is expressed as picomoles of conjugate per milligram of membrane protein.

The kinetics of nonenzymatic QO-SG formation was determined spectrophotometrically at 37 °C as described above. Reactions mixtures contained 0.1 M potassium phosphate (pH 7.5), 60 μM NQO, and 2.5−7 mM GSH. Data were fitted to the first-order rate equation to determine pseudo-first-order rate constants from which the second-order rate constant, k_2, was calculated.

RESULTS

MRP1-Mediated Transport of QO-SG. To determine the efficiency and capacity of MRP1 to mediate transport of QO-SG, the kinetics of QO-SG uptake into inside-out membrane vesicles derived from MCF7 cells was determined. As shown in Figure 1, vesicles derived from MRP1-positive (MCF7/MRP1−10) but not MRP1-negative (MCF7/WT) cells supported robust ATP-dependent transport of QO-SG. Kinetic constants were calculated from initial velocity data (parts A and B of Figure 2, ●) and include a K_m of 9.5 μM. The V_{max} for QO-SG transport, 250 pmol min^{-1} mg^{-1}, is considerably higher than the V_{max} obtained for another good MRP1 substrate, monoglutathionyl chlorambucil (82 pmol min^{-1} mg^{-1}), using a similar vesicle preparation (*16*). Overall, because of its higher K_m, the efficiency of QO-SG transport ($V_{max}/K_m \sim 0.03$ mL min^{-1} mg^{-1}) is about 7−8-fold lower than that of monoglutathionyl chlorambucil.

GSH has been shown to stimulate the transport of several MRP1 substrates (*22*−*28*). This stimulation occurs by mechanisms that may involve cotransport of GSH with the substrate, modulation of MRP1 activity (presumably an allosteric effect), or both. As shown in Figure 2A (■) and Table 1, inclusion of 5 mM GSH significantly augments the V_{max} of QO-SG transport. Similarly, 5 mM *S*-methyl glutathione stimulates the rate of QO-SG transport, indicating that the free thiol of GSH is not required for this augmentation (Figure 2B, ■, and Table 1). Additionally, inclusion of GSH or its *S*-methyl analogue was associated with an increase in the apparent K_m of MRP1-mediated transport (Table 1). To determine whether the action of GSH and its *S*-methyl analogue on MRP1-mediated QO-SG transport involves cotransport of GSH or an alternative mechanism, we examined the effect of unlabeled QO-SG on ATP-dependent ^{3}H-GSH transport. Vesicles containing MRP1 supported ATP-dependent transport of 100 μM GSH (244 ± 6 pmol/mg in 20 min) that was considerably greater than the transport by MRP1-minus vesicles (33 ± 6 pmol/mg in 20 min). As shown (Figure 3), addition of QO-SG did not stimulate the basal level of ^{3}H-GSH uptake into inside-out MRP1-containing membrane vesicles. In fact, addition of QO-SG resulted in a concentration-dependent inhibition of ^{3}H-GSH uptake over the full range of QO-SG concentrations (0.5−50 μM) used. These data suggest a mechanism for GSH-enhanced, MRP1-mediated QO-SG transport that does not involve cotransport of GSH and may apply generally to the transport of other GSH conjugates (see the Discussion). With respect to the cellular consequences of MPR1 expression, we conclude that MRP1 mediates efficient, GSH-stimulated efflux transport of QO-SG and is therefore well-suited to detoxify QO-SG formed intracellularly.

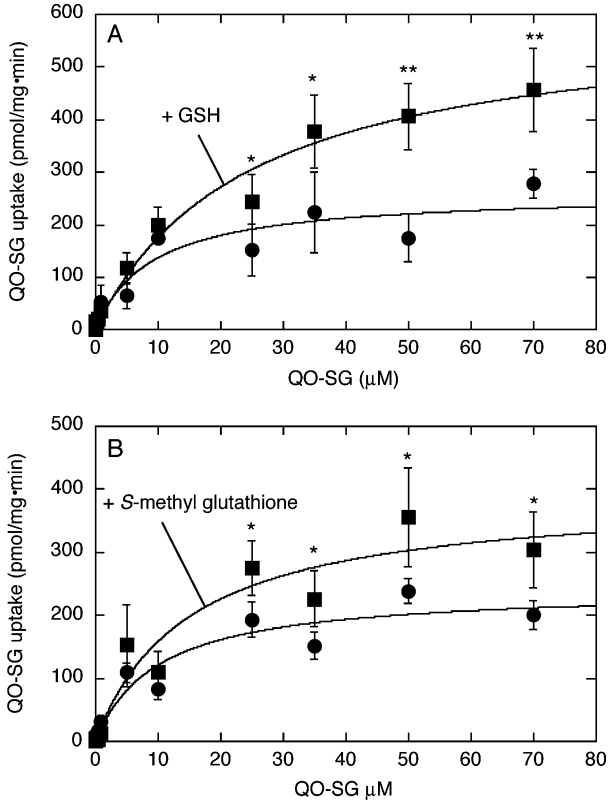

FIGURE 2: Kinetics of MRP1-mediated QO-SG transport: GSH and *S*-methyl glutathione augment initial velocities. Initial velocities of MRP1-dependent uptake are illustrated as a function of the substrate (^{3}H-QO-SG) concentration in the absence (●) or presence (■) of 5 mM GSH (A) or 5 mM *S*-methyl glutathione (B). Values are the means of four determinations ± 1 standard deviation (SD). Data were analyzed by an unpaired two-tailed Student's *t*-test; asterisks indicate $p < 0.05$ (*) or $p \leq 0.005$ (**).

Table 1: ATP- and MRP1-Dependent Transport of QO-SG Alone or in the Presence of 5 mM GSH or *S*-Methyl GSH

	K_m^{app} (μM)	V_{max}^{app} (pmol min^{-1} mg^{-1})
QO-SG	9.5	250
QO-SG (+GSH)	24	600
QO-SG (+*S*-methyl glutathione)	15	390

Role of MRP1 in NQO Transport? Previously, we observed that coexpression of MRP1 and GSTP1a-1a conferred a high level of resistance to NQO toxicity (*15*). Surprisingly,

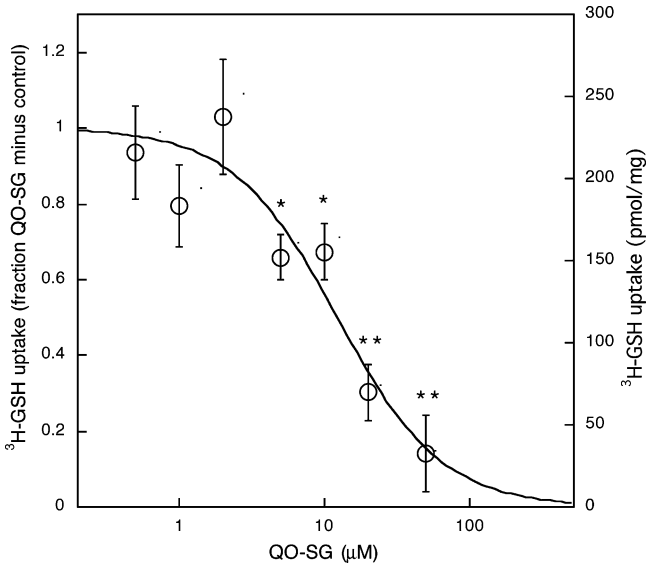

FIGURE 3: QO-SG inhibits ATP-dependent transport of ^{3}H-GSH by MRP1. Uptake of ^{3}H-GSH into membrane vesicles prepared from MRP1-positive (MRP1−10) MCF7 cells was measured as described in the Experimental Procedures using 100 μM ^{3}H-GSH. Shown are mean values ($n = 4$; ±1 SEM) of ATP-dependent uptake of ^{3}H-GSH (and 0−50 μM QO-SG) over the incubation period of 20 min expressed as a proportion of minus QO-SG controls (left axis) or picomoles of uptake per milligram of vesicle protein (right axis). * and ** signify $p < 0.03$ and $p < 0.001$, respectively (Student's t test).

however, expression of MRP1 alone (without GSTP1a-1a), conditions where the rate of QO-SG formation is dramatically reduced, resulted in a modest but measurable level of cellular resistance to NQO (1.5−4-fold). This suggested that NQO itself may be a substrate of MRP1. Indeed, many neutral or cationic lipophilic compounds are substrates of MRP1 (*23*, *25*, *27*). For these substrates, transport is generally GSH-dependent even though no stable covalent adducts are formed with GSH. The possibility that MRP1 mediates transport of unmodified NQO was investigated in the following series of experiments.

Studies shown in Figure 4A examined whether unlabeled NQO, acting as a potential competitive substrate, could inhibit the MRP1-mediated transport of ^{3}H-QO-SG. Indeed, using 1 μM ^{3}H-QO-SG, transport was significantly inhibited by 50 μM NQO but only in the presence of GSH (5 mM). Moreover, NQO (50 μM) stimulated the MRP1-associated uptake of ^{3}H-GSH (Figure 4B). Together, these data are consistent with the possibility that NQO is transported by MRP1 in a GSH-dependent manner. To verify this possibility, transport of ^{3}H-NQO was examined. MRP1-dependent transport of ^{3}H-NQO was observed in the presence of 5 mM GSH (parts A and B of Figure 5); however, no transport was apparent in the absence of GSH or using vesicles derived from MRP1-minus cells (Figure 5A). The importance of the free thiol in GSH-dependent transport of NQO was evaluated using the *S*-methyl analogue of glutathione. As shown in Figure 6A, whereas GSH supports ^{3}H-NQO transport, *S*-methyl glutathione does not. As a control, transport of a known GSH-dependent MRP1 substrate, estrone-3-sulfate, was examined. Both GSH and *S*-methyl glutathione stimulated MRP1-dependent uptake of radiolabeled estrone-3-sulfate (Figure 6B). Thus, MRP-mediated transport of NQO is clearly distinguished from estone-3-sulfate in that the free

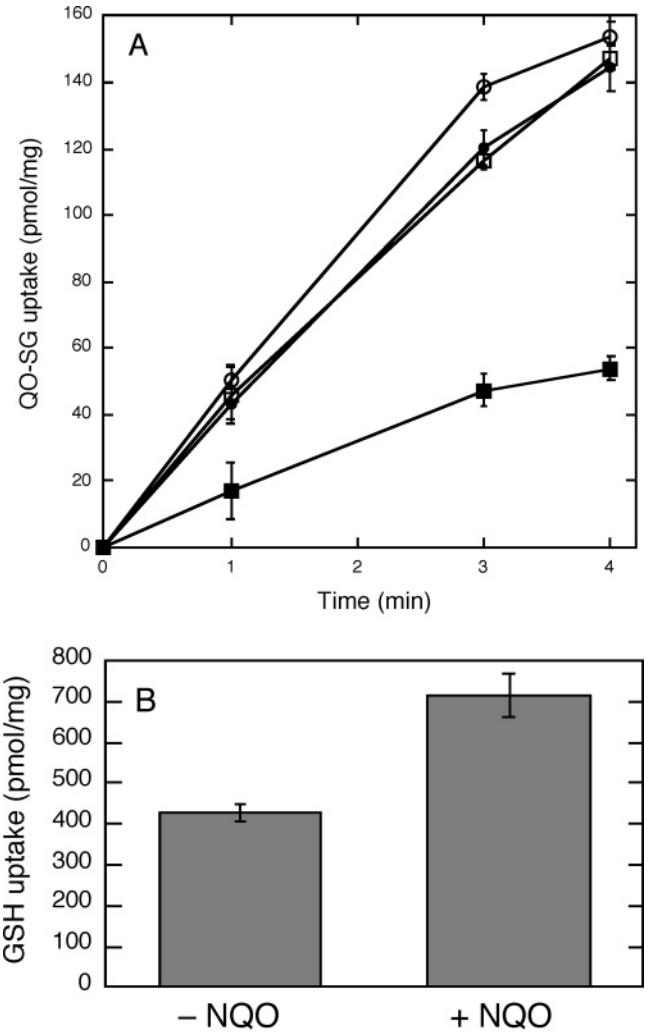

FIGURE 4: NQO mediates GSH-dependent inhibition of ^{3}H-QO-SG transport and stimulates ^{3}H-GSH transport, by MRP1-containing inside-out membrane vesicles. (A) Shown is the ATP-dependent uptake of ^{3}H-QO-SG into inside-out membrane vesicles derived from MRP1-positive (MCF7/MRP1−10) cells. ^{3}H-QO-SG (1 μM) was incubated in the absence of NQO and GSH (○), presence of 5 mM GSH (●), presence of 50 μM NQO (□), or presence of 50 μM NQO *and* 5 mM GSH together (■). Data points are expressed as picomoles per milligram of membrane protein and represent mean values of triplicate determinations ± 1 SD. (B) Uptake of ^{3}H-GSH into membrane vesicles prepared from MRP1-containing cells was measured in the presence or absence of 50 μM NQO exactly as described in Figure 3 and the Experimental Procedures. Data are expressed as the transport of ^{3}H-GSH (picomoles per milligram of membrane protein in 20 min); bars represent mean values of triplicate determinations ± 1 SD.

thiol of GSH is required for NQO but not for stimulation of estrone-3-sulfate transport. Moreover, inspection of the NQO transport curves at early time points (0−4 min, Figures 5B and 6A) reveals a reproducible time lag in the rate of NQO uptake, indicating that a time- and GSH-dependent modification of the substrate is necessary for efficient transport. This view suggests that the transported substrate might be QO-SG, formed during the transport reaction incubations, and not NQO. While vesicles contained no contaminating GST activity toward NQO (not shown), incubation of 90 μM NQO with 5 mM GSH ± vesicles (but under conditions otherwise identical to transport studies) resulted in the formation of ~70 pmol of QO-SG/50 μL of reaction in 4 min. Under the same conditions but with the inclusion of inside-out mem-

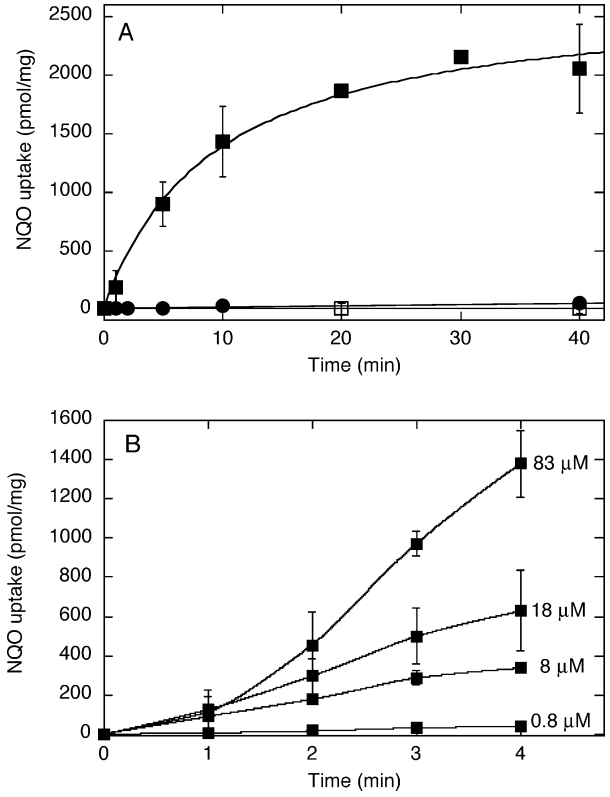

FIGURE 5: GSH-dependent transport of NQO by MRP1. Shown is ATP-dependent uptake of ^{3}H-NQO into inside-out membrane vesicles derived from MRP1-minus (MCF7/WT, □) or MRP1-positive (MCF7/MRP1−10, ● and ■) cells. Transport was measured as described in the Experimental Procedures in the absence (●) or presence (■) of 5 mM GSH. Data points represent mean values (^{3}H-NQO transported per milligram of membrane protein) from duplicate experiments; error bars show the range of values. The concentrations of ^{3}H-NQO added were 12 μM in (A), the 40 min time course, or 0.8−83 μM as indicated in (B), the 4 min time course.

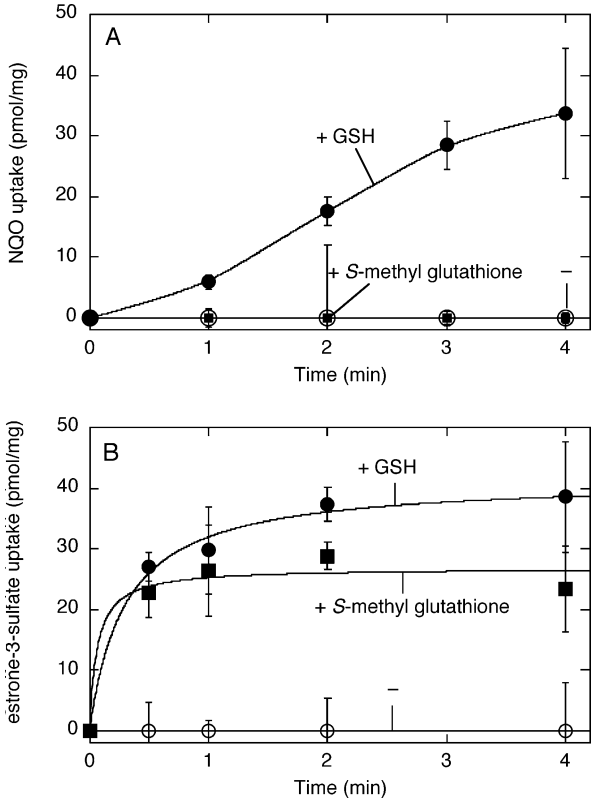

FIGURE 6: *S*-Methyl glutathione does *not* support MRP1-mediated transport of NQO. Shown is the ATP-dependent uptake of 1 μM ^{3}H-NQO (A) or 300 nM ^{3}H-estrone-3-sulfate (B) into vesicles prepared from MRP1-positive (MCF7/MRP1−10 cells). Transport was measured in the absence of additions (○) or, alternatively, in the presence of 5 mM GSH (●) or 5 mM *S*-methyl glutathione (■) as described (Experimental Procedures). Values represent the means of triplicate determinations ± 1 SD.

brane vesicles, MRP1 supported uptake of ∼35 pmol of ^{3}H-NQO. These data suggest that MRP1-mediated, GSH-dependent transport of ^{3}H-NQO can be fully attributed to uptake of QO-SG formed nonenzymatically. In aggregate, our results indicate that MRP1-associated protection in cells that lack GST is a consequence of the transport of QO-SG formed nonenzymatically and that MRP1-mediated transport of unmodified NQO is unlikely.

Role of GSTP1a-1a in NQO Detoxification. The recognition that nonenzymatic QO-SG formation is significant under physiological conditions and concentrations of GSH raises questions about the quantitative role of GSTP1a-1a in overall NQO detoxification. To address this issue, we examined the kinetics of QO-SG formation at pH 7.5, 37 °C, and physiological levels of GSH. At 5 mM GSH and in the absence of GST, QO-SG formation assumes an excellent fit to pseudo-first-order kinetics (Figure 7, QO-SG formation expressed as the disappearance of NQO). From these data and pseudo-first-order rate constants also determined from experiments using 2.5 and 7 mM GSH, a second-order rate constant, k_2, of 0.17 M^{-1} s^{-1} was calculated. Using similar conditions, the kinetics of GSTP1a-1a catalyzed QO-SG formation was examined (inset of Figure 7), yielding a k_{cat}/K_m of 4.7 × 10^5 M^{-1} s^{-1}. The enzymatic rate enhancement factor, $(k_{cat}/K_m)/k_2$, at 2.8 × 10^6, is among the highest for any cytosolic GST/substrate pair reported.

DISCUSSION

Previous studies have shown that MRP1 and MRP2 confer resistance to the geno- and cytotoxicities of the electrophilic carcinogen, NQO (*15*, *29*). MRP-associated resistance was greatly enhanced by the coexpression of GSTP1a-1a, suggesting that MRP1/2 operate by supporting efflux of the GSH conjugate of NQO, QO-SG. Analyses described in the present study establish that MRP1 is an efficient, ATP-dependent transporter of QO-SG with a K_m of 9.5 μM and a V_{max} comparable to other good substrates of MRP1; thus, MRP1 is kinetically well-suited to detoxify conjugates of NQO formed intracellularly.

Roles of GSH and MRP1. As with many MRP1 substrates, the rate of MRP1-mediated QO-SG transport is augmented by GSH. This emphasizes the multifaceted importance of GSH in detoxification. For NQO, conjugation with GSH renders the electrophile less reactive *and* tags the derivative, QO-SG, for efficient MRP1-mediated efflux disposal. Moreover and independently of conjugation, GSH enhances the rate of QO-SG transport, especially at higher QO-SG concentrations where the cell is under particularly toxic stress. While GSH-stimulated transport is a property shared by other MRP1 substrates such as estrone-3-sulfate and vincristine (*22*−*24*, *26*), there are features of GSH stimulation of QO-SG transport that are clearly distinguishable from its stimulation of these other substrates, features that may apply generally to the transport of GSH conjugates and that suggest

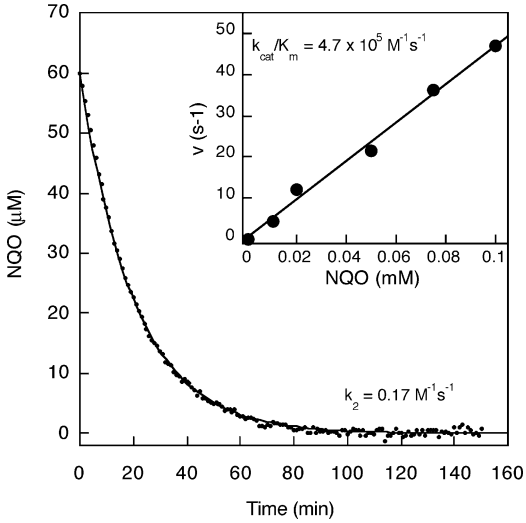

FIGURE 7: Nonenzymatic and GSTP1a-1a-catalyzed QO-SG formation. Shown are the kinetics of nonenzymatic and GSTP1a-1a-catalyzed (inset) QO-SG formation. Reactions were accomplished under physiological temperature (37 °C), pH 7.5, and GSH (2.5−7.5 mM) as described in the Experimental Procedures. (Nonenzymatic reactions) The reaction shown contained 5 mM GSH and was initiated with 60 μM NQO. The time-dependent consumption of NQO substrate was fitted to pseudo-first-order kinetics. From pseudo-first-order rate constants derived from these data and similar reactions using 2.5 and 7.5 mM GSH, the second-order rate constant, k_2, was calculated. (GSTP1a-1a-catalyzed reactions) The inset shows initial velocities, v (s^{-1}), plotted as a function of the NQO concentration. From these data, k_{cat}/K_m was estimated (Experimental Procedures).

mechanisms by which GSH mediates its transport-enhancing effects.

It is informative to consider three classes of MRP1 substrates for which transport is influenced by GSH or its *S*-alkyl analogues: organic anion substrates that are GSH conjugates, represented by QO-SG; organic anion conjugate substrates that are not GSH conjugates, represented by estrone-3-sulfate; and cationic (or neutral) lipophiles that are not conjugates, represented by vincristine. MRP1-mediated transport of all three is stimulated by *S*-methyl glutathione, as well as by GSH, indicating that the reactive-free thiol is dispensable for transport augmentation. However, transport of these substrates is distinguishable in that GSH (or one of its analogues) is essential for transport of vincristine but not the other two substrates. Indeed, QO-SG transport is quite efficient in the absence of GSH even though rates are significantly enhanced by physiological levels of GSH (5 mM), especially at high QO-SG concentrations. The GSH dependence of estrone-3-sulfate lies between that of vincristine and QO-SG: while GSH is not essential for transport, addition of millimolar GSH stimulates the rate and efficiency of estrone-3-sulfate transport severalfold (*26*). Together, these results are consistent with mechanisms of MRP1-mediated transport proposed by others that, in general, hold that MRP1 contains a bi- or multipartite binding pocket for substrate transport (*23*, *30*−*32*).

This substrate-binding pocket is thought to contain an element that accommodates the lipophilic substrate or its lipophilic moiety and another element that accommodates GSH or the anionic moiety of the substrate. It is believed that both sites must be occupied to support efficient ATP-dependent transport. For the lipophilic substrate, vincristine, there is considerable evidence that GSH and vincristine are cotransported by MRP1; in particular, vincristine stimulates a large increase, above basal levels, in ^{3}H-GSH transport (*24*). In contrast, although we observed little GSH-independent estrone-3-sulfate transport, other studies indicate that the sulfate moiety on estone-3-sulfate is sufficient for MRP1-mediated transport (*22*, *26*). While GSH greatly stimulates estone-3-sulfate transport, this stimulation does not appear to involve cotransport of GSH because estrone-3-sulfate has, at best, a very small effect on basal ^{3}H-GSH uptake (*26*), an effect observed only at very high estrone-3-sulfate concentrations (more than 20-fold > K_m). For QO-SG where GSH stimulation of transport is less pronounced, there is no reciprocal stimulation of ^{3}H-GSH transport; instead, inclusion of QO-SG results in a concentration-dependent inhibition of ^{3}H-GSH transport. The reciprocal effects of GSH and QO-SG on their transport by MRP1 are remarkably analogous to those reported for the MRP1 substrate pair, the *O*-glucuronide of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL-*O*-G) and leukotriene C_4 (LTC_4), by Leslie et al. (*19*). In these studies, MRP1-mediated transport of NNAL-*O*-G was inhibited by LTC_4 (*19*), much as GSH transport is inhibited by QO-SG (Figure 3). In the reciprocal experiments, both the V_{max} and K_m of NNAL-*O*-G transport were increased by LTC_4 (*19*), similar to the pattern observed for GSH (and *S*-methyl glutathione) modulation of QO-SG transport (Figure 2 and Table 1).

Consistent with our data is a model that incorporates the existence of at least two functionally distinguishable GSH-interaction sites on MRP1. The model incorporates elements proposed by several groups (*31*, *33*, *34*) and is particularly similar to the two-site ligand/substrate-binding model proposed by Zelcer et al. and Chu et al. to explain organic anion regulation of estradiol-17-β-D-glucuronide transport by MRP2 (*35*, *36*). According to this view, one site lies within the substrate-binding pocket and, for substrate transport, must be occupied either by free GSH (or GSH analogues) or by the anionic moiety (e.g., glutathionyl, sulfate, or glucuronosyl) of an amphiphilic substrate. For lipophilic cation substrates such as vincristine, co-transport of unconjugated GSH bound to the substrate-binding pocket occurs, whereas for conjugates such as estrone-3-sulfate and QO-SG, cotransport of free GSH is not required (if even possible) for conjugate transport. For the transport of anionic conjugates, the role of GSH might be to alter MRP1 transport activity by a functionally distinct interaction with MRP1. These distinct modulatory interactions of GSH with MRP1 may occur at a site distant from the transport-associated substrate-binding pocket, or alternatively, GSH, when acting as a transport-regulating ligand, may bind within the substrate-binding pocket in such a manner that it influences transport of a second substrate but is itself not transported.

Indeed, support for such a modulatory role of GSH is provided by the finding that GSH confers a simultaneous ~6-fold decrease in K_m^{app} and ~4-fold increase in V_{max}^{app} to MRP1-mediated transport of estrone-3-sulfate (*26*). The situation for QO-SG is somewhat different; the inclusion of 5 mM GSH or *S*-methyl glutathione is associated with a 2.4-fold or 1.6-fold, respectively, increase in V_{max}^{app} but a simultaneous increase, rather than decrease, in K_m^{app}. This discrepancy between the effect of GSH on the modulation of

QO-SG versus estrone-3-sulfate transport is explained if, in the transport-associated substrate-binding pocket, the binding element for the glutathionyl moiety of QO-SG completely overlaps with the binding element for free GSH, whereas overlap is absent or incomplete between sites for the sulfate moiety of estrone-3-sulfate and GSH. In this case, estrone-3-sulfate would not interfere with basal GSH transport. In contrast, GSH (low-affinity substrate, in mM K_m) and QO-SG (high-affinity substrate, in μM K_m) act as competing substrates at the substrate-binding pocket. These interactions at the substrate-binding pocket, distinct from GSH interactions at the modulatory site, would explain why QO-SG, acting as a competitive inhibitor, reduces ^{3}H-GSH transport. Moreover, this model explains how GSH can both stimulate QO-SG transport (increase V_{max}), via interacting with the modulatory site, and increase the K_m^{app}, by acting as a weak competitive inhibitor at the region of the substrate-binding pocket associated with transport. It should be emphasized that this model may also apply to other transport-regulating ligands of MRP1 besides GSH, ligands that may interact with the same modulatory site on MRP1, and is not meant to imply there are necessarily unique modulatory binding sites for each of these ligands.

Role of GSTP1a-1a. Our previous studies indicated that expression of MRP1 alone, in the absence of GST, could confer low-level resistance to NQO toxicities (*15*), despite reports that QO-SG formation is quite slow in the absence of GST (*14*, *20*). Accordingly, we examined the possibility that unmodified NQO was also a substrate of MRP1. While, ATP-dependent transport of NQO was demonstrated, this MRP1-mediated process was absolutely dependent upon GSH. In contrast to studies using QO-SG and estrone-3-sulfate, *S*-methyl glutathione could not substitute for GSH, indicating that the reactive free thiol of GSH is required for MRP1-mediated transport of NQO. Moreover, additional studies revealed that significant QO-SG is formed nonenzymatically over the time frames used in both transport (this study) and cellular experiments (*15*, *29*). Quantitative analyses of experiments showed that MRP1-mediated uptake of ^{3}H-NQO could be entirely attributed to QO-SG formed nonenyzmatically during the transport experiments. Thus, in GST-deficient cells, MRP1-associated protection is most likely due to the removal of QO-SG formed nonenzymatically. The argument for MRP1-mediated transport of unmodified NQO is not supported by the data.

Finding that nonenzymatic QO-SG formation occurs at rates higher than expected prompted a reassessment of the relative role of GSTP1a-1a catalysis in NQO detoxifications at physiological temperature, pH, and GSH levels. These results confirmed that GSTP1a-1a is a highly efficient catalyst of QO-SG formation ($k_{cat}/K_m = 4.7 \times 10^5$ M^{-1} s^{-1}) with a rate enhancement factor, $(K_{cat}/K_m)/k_2$, of 2.8×10^6, a very high value for a GST isozyme/substrate pair (*21*). These results underscore the dominant role played by GSTP1a-1a, when expressed, over nonenzymatic QO-SG formation in the detoxification of NQO; they explain the requirement for GSTP1a-1a to achieve the high-level MRP1-associated NQO detoxification observed in model cell lines.

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