Conjugation of Haloalkanes by Bacterial and Mammalian Glutathione Transferases: Mono- and Dihalomethanes

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Conjugation of Haloalkanes by Bacterial and Mammalian Glutathione Transferases: Mono- and Dihalomethanes

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A primary route of metabolism of dihalomethanes occurs via glutathione (GSH) transferase-catalyzed conjugation. Mammalian theta class GSH transferases and a group of bacterial dichloromethane dehalogenases are able to catalyze the hydrolytic dehalogenation of dihalomethanes via GSH conjugation and subsequent formation of HCHO. Dihalomethanes have been shown to induce revertants in *Salmonella typhimurium* TA 1535 expressing theta class GSH transferases. Two mammalian theta class GSH transferases (rat GST 5-5 and human GST T1) and the bacterial dehalogenase DM11 were compared in the in vitro conjugation of CH_3Cl and using in vitro assays (HCHO formation) and the *S. typhimurium* mutagenesis assay with the dihalomethanes CH_2Cl_2, CH_2Br_2, CH_2BrCl, CH_2ICl, CH_2I_2, and CH_2ClF. GSTs 5-5 and T1 had similar characteristics and exhibited first-order rather than Michaelis−Menten kinetics for HCHO formation over the range of dihalomethane concentrations tested. In contrast, the DM11 enzyme displayed typical hyperbolic Michaelis−Menten kinetics for all of the compounds tested. A similar pattern was observed for the conjugation of CH_3Cl. The reversion tests with *S. typhimurium* expressing DM11 or GST 5-5 showed a concentration-dependent increase in revertants for most of the dihalomethanes, and DM11 produced revertants at dihalomethane concentrations lower than GST 5-5. Collectively, the results indicate that rates of conversion of dihalomethanes to HCHO are not correlated with mutagenicity and that GSH conjugates are genotoxic. The results are compared with the conjugation and genotoxicity of haloethanes in the preceding paper in this issue [Wheeler, J. B., Stourman, N. V., Armstrong, R. N., and Guengerich, F. P. (2001) *Chem. Res. Toxicol.* **14**, 1107−1117]. The halide order appears most important in the dihalomethane conjugation reactions catalyzed by GST 5-5 and less so in GST T1 and DM11, probably due to changes in the rate-limiting steps.

Introduction

Halomethanes constitute a diverse group of halogenated hydrocarbons that have been of considerable interest for many years because of their biological properties, including toxicity (*1*, *2*). Although halomethanes are often only considered as industrial chemicals, they are also natural products. Global production of CH_3Cl from natural sources is 3−8 million tons/year, and CH_3Br is also a natural product (*3*). Industrially, CH_3Cl is used as an aerosol propellant, dewaxing agent, and blowing agent in molding polystyrene and polyurethane foams (*2*, *4*).

Of the dihalomethanes, the most prominent is CH_2Cl_2. It is commonly used in paint stripping, urethane foam production, solvent extraction of food, as a degreaser, and as an organic solvent in the synthesis of plastics, pharmaceuticals, and other chemicals (*5*, *6*). Recent estimates indicate that annual production in the United States is 2×10^8 kg (*7*) and that 238 000 (U.S.) workers are exposed to CH_2Cl_2 (*6*). The 8-h time-weighted permissible exposure limit was lowered from 500 to 25 ppm, primarily because of concern about rodent tumorigenicity of the compound (*6*, *7*). Mice exposed to 2000 ppm CH_2Cl_2 (by inhalation) for ≥26 weeks developed lung and liver tumors in the apparent absence of cytotoxicity and sustained cell proliferation (*8*). CH_2Cl_2 can also produce tumors in the mammary gland, skin, neck, and salivary glands in rats (*8*) although rats and hamsters appear to be much more resistant to the toxic effects of CH_2Cl_2 (*9*−*12*).

We have been interested in the metabolism of haloalkanes for some time, particularly in the conjugation by GSH transferases (*13*). In general, GSH conjugation of haloalkanes is a detoxication reaction (e.g., for mono-

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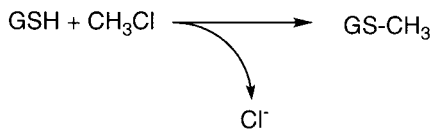
Scheme 1. Pathway for Conjugation of CH_3Cl with GSH



haloalkanes) but numerous exceptions are known (*13*−*16*). In an in vivo human study it has been previously shown that the theta class GSH transferase (GST)[1] GST T1 is the only enzyme responsible for metabolism of CH_3Cl in humans (Scheme 1) and that the extent of conjugation of CH_3Cl is directly related to the GST T1 phenotype (*17*).

The modeling of CH_2Cl_2 tumor data in mouse and rat to human risk assessment has been considered several times (*11*, *18*−*21*). Inasmuch as CH_2Cl_2 appears to be biologically inert (except for anesthetic action) the observed tumorigenicity is accepted to be due to metabolites of CH_2Cl_2. Therefore, a key issue in human toxicity has been comparison of the extent of metabolic activation and detoxication of the compound. The two major routes for the metabolism of CH_2Cl_2 (and other dihalomethanes) are oxidation and conjugation with GSH (*22*, *23*). The oxidation pathway yields CO (via formyl chloride) (*24*) while conjugation yields (putative) *S*-(chloromethyl)GSH that is hydrolyzed to $GSCH_2OH$ and then HCHO (Scheme 1). The conjugation pathway was proposed to be related to the tumorigenicity of CH_2Cl_2 because the dose dependence of tumor production was not saturable, similar to the in vitro GSH conjugation of CH_2Cl_2 (*11*, *18*, *19*). Unlike the one-carbon compounds discussed here, there is no evidence that tumor formation in rodents is nonsaturable for the two-carbon compounds discussed previously (*25*). The role of GSH conjugation of CH_2Cl_2 in mutagenicity of dihalomethanes was difficult to demonstrate because of the presence of GSH and GSH transferases in the bacteria used in the test systems (*26*−*29*). Heterologous expression of mammalian theta class GSH transferases in *Salmonella typhimurium* and *Escherichia coli* systems has been used to demonstrate the role of GSH conjugation in the genotoxicity of dihalomethanes (*15*, *30*, *31*).

A number of questions exist about the mechanism of CH_2Cl_2 tumorigenicity (*32*). The results of several investigations indicate that the tumors do not develop due to cell proliferation, providing a contrast with $CHCl_3$ (*33*). The most generally accepted explanation of the carcinogenicity is genotoxicity, and the limited number of point mutations that have been analyzed appear to support this conclusion (*34*, *35*). Heck has presented the hypothesis that the HCHO formed in the reaction is responsible for the tumorigenicity due to formation of DNA−protein cross-links (*36*). The addition of HCHO to bacteria does not cause base pair mutations (*15*, *29*), but the possibility exists that HCHO formed within cells might have different biological properties. Physiologically based pharmacokinetic modeling based on metabolic information derived from experimental animal studies predicts a much lower risk from CH_2Cl_2 for humans than mice (*11*). However, the variation in both the oxidative and conjugation pathways in humans is considerable because of the variability of P450 2E1 and the polymorphic GST T1 (*20*, *21*).

To define some of the aspects of the toxicity of CH_2Cl_2 and other dihalomethanes, we extended our earlier investigations with GSH transferases (*15*, *30*, *31*, *37*) to a series of different dihalomethanes and enzymes, including a bacterial GSH transferase from strain *Methylophilus* sp. *DM11* that enables these bacteria to grow on CH_2Cl_2 as a carbon source (*38*, *39*). The enzyme DM11 is similar to rat GST 5-5 and human GST T1 in its ability to conjugate dihalomethanes, resulting in the production of HCHO (*39*). This bacterial enzyme (DM11), rat GST 5-5, and human GST T1 were purified and steady-state kinetic parameters were measured for the conjugation of a series of six dihalomethanes. The GSH transferases were also expressed within the mutagenicity tester strain *S. typhimurium* TA1535 (*15*) and the results were compared to the conjugation of the dihalomethanes by the purified enzymes. The results are considered in terms of comparisons of the three enzymes and the influence of the halogen atoms, in the context of these results with 1,2-dihaloalkanes (*25*).

Experimental Procedures

Reagents and Chemicals. CH_2ClF was purchased from Flura Corporation (Newport, TN). The remaining dihalomethanes and all other reagent grade chemicals were purchased from Aldrich (Milwaukee, WI). Agar, nutrient broth, and other Ames test reagents were obtained from VWR (South Plainfield, NJ).

GSH Transferase Expression Constructs. GST 5-5 and GST T1 were expressed in pKK233-2 based *E. coli* systems and purified as described previously (*25*, *40*). Human GST T1 with C-terminal pentahistidine tag (GST T1h) and DM11 were expressed and purified as described previously (*25*). Antibodies to DM11 and GSH transferases were prepared and used as described previously (*25*).

CH_3Cl Depletion Assays. A reaction mixture (3.0 mL) containing 0.10 M potassium phosphate buffer (pH 7.3), 7.5 mM GSH, and 0.16−0.24 μM GSH transferase (GST T1 or DM11) was incubated for 5 min at 37 °C with mixing in a sealed 9 mL vial. Prior to injecting a stock concentration (2.2×10^4 ppm) of CH_3Cl, an equivalent amount of air was withdrawn from a vial. At various time-points, 0.5 mL of gas from the vial was withdrawn and injected into the gas chromatograph. Analysis was performed on a Shimadzu GC8A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a 0.25 mL injection loop, flame ionization detector, and a CP Sil 5CB column (0.52 mm × 50 m) using N_2 as a carrier gas at an operating pressure of 0.7 kg/cm^2. The injector and column temperatures were 200 and 100 °C, respectively. A set of CH_3Cl standards were used for calibration of the data (varying concentrations of CH_3Cl in the absence of a liquid phase at 37 °C). A partition coefficient of 2:1/liquid:gas was measured based on the standards compared to the control vials, which contained reaction buffers and other components but not enzyme. After each gas sample was removed for analysis, pressure within the vials was allowed to equilibrate for a few seconds before the vials were returned to the incubator. A background rate of CH_3Cl depletion caused by the removal of a portion of the gas phase at each time point was determined with a reaction mixture lacking enzyme. No significant nonenzymatic GSH reaction with CH_3Cl occurred within 2 h.

Assays of HCHO Formation. *(1) Nash Assay.* Purified enzyme or cytosol isolated from *S. typhimurium* was used to estimate HCHO using a Nash assay (*41*, *42*). Unless otherwise stated, the standard reaction mix contained 100 mM Tris-SO_4 (pH 8.0), 7.5 mM GSH, dihalomethane, and purified protein in

[1] Abbreviations: GST, GSH transferase (designating a specific enzyme); GST T1h, human GST T1 with C-terminal pentahistidine tag.

a final volume of 0.6 mL. Solutions of dihalomethanes were prepared and used as stocks based on published concentrations of saturated solutions in water at 25 °C (*43*). All reactions were performed at 37 °C in capped 1.5 mL polypropylene vials. Reactions were started with the addition of dihalomethane and quenched with 0.4 mL of 8% $HClO_4$. Nash reagent (*41*, *42*) (0.2 mL) was added to the quenched reactions, and the mixtures were incubated at 60 °C for 20 min. The absorbance at 412 nm of each sample was measured. Standard curves were prepared using dilutions of a 37% HCHO stock solution (Aldrich).

(2) Formaldehyde Dehydrogenase Assay (44, 45). The procedure was performed as described elsewhere (*45*). Analysis was done at 37 °C in a 1.0 mL cuvette equipped with a Teflon stopper. Reagent concentrations were as follows: 100 mM potassium phosphate (pH 8.0), 1 mM NAD^+, 1 unit (0.23 mg) of formaldehyde dehydrogenase/mL (EC 1.2.1.46, from *Pseudomonas putida*, Sigma Chemical Co., St. Louis, MO), 0.27 μM GST 5-5, and 3 mM CH_2Br_2. GSH concentrations were varied from 0 to 7.5 mM, and the assay was started by the addition of CH_2Br_2. Analysis was done using an OLIS/Cary 14 (On-Line Instrument Systems, Bogart, GA) or a Perkin-Elmer Spectrometer Lambda 18 (Norwalk, CT) spectrophotometer and the rate of HCHO formation was determined using linear regression to fit the initial rate of NADH production based on the change in absorbance at 340 nm.

Mutagenesis Experiments. Ames reversion assays were performed as previously described (*46*). DM11 was expressed using the pTrc99A plasmid, and GSTs 5-5 and T1 were expressed using the pKK233-2 plasmid. In the dihalomethane studies, 100 μL of *S. typhimurium* culture was mixed with 100 μL of the mutagen in 0.2 M sodium phosphate buffer (pH 7.4) for 5 min at 37 °C. The solutions were then mixed with 2 mL of top agar and plated on minimal glucose plates with 50 μg of ampicillin/mL. Solutions of dihalomethanes were prepared based on published concentrations of saturated solutions in water at 25 °C (*43*) and were diluted into sterile phosphate buffer.

Analysis. Linear and nonlinear regression analysis were calculated using Prism 3 (Graphpad Software, San Diego, CA)

Results

***K_m* for GSH.** An initial concern in all the work done with halomethanes using the three GSH transferases was the selection of an appropriate fixed concentration of GSH. DM11 has been reported to have $K_{m,GSH}$ values of 66−82 μM (*47*, *48*). The $K_{m,GSH}$ of rat GST 5-5 has been reported to be ~60 μM with CH_2Cl_2 (*47*, *49*) and CH_2ClF (*47*) but 1210 μM for CH_2Br_2 (*47*). We reexamined the CH_2Br_2 issue with GST 5-5 because saturation with GSH would require a very high concentration of GSH, significantly greater than prevailing under physiological conditions. A value of 156 ± 21 μM was measured (see Supporting Information). On the basis of this value, we used a GSH concentration of 5 or 7.5 mM with all GSH transferases in this study. Concentrations for dihalomethanes were either 1 mM for DM11 or 7.5 mM for GSTs 5-5 and T1.

CH_3Cl Depletion Assays. Conjugation of CH_3Cl by rat and human theta class GSH transferases has been demonstrated in human erythrocytes (*50*) (Scheme 1). Purified DM11, rat GST 5-5, and human GST T1h were compared in a substrate depletion assay, in which CH_3Cl was measured in the gas phase at various concentrations over a period of 60 min. A linear, nonsaturating trend over the concentrations evaluated was observed for rat GST 5-5 and human GST T1h (Figure 1). In contrast, DM11 showed an increase in reaction velocity at lower concentrations of CH_3Cl. The velocity began to decrease when the concentration in the liquid-phase reached 150 μM.

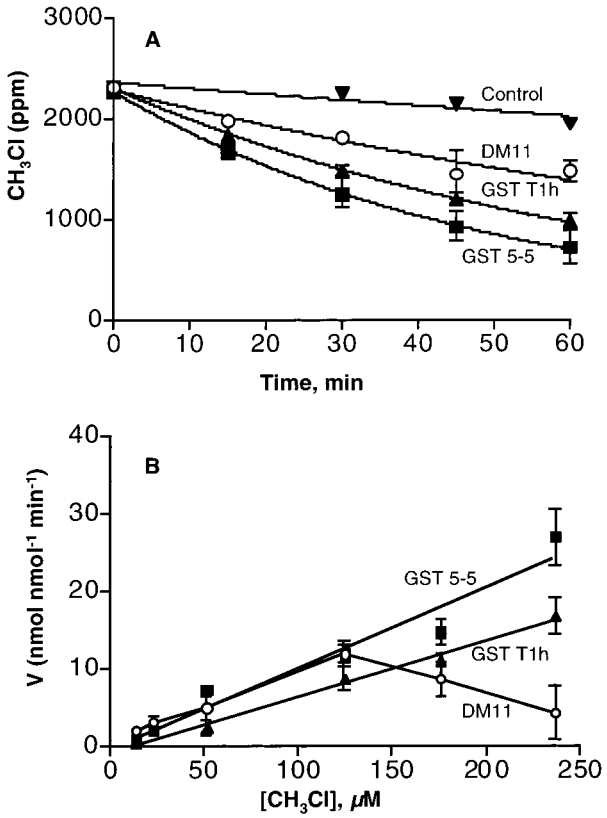


Figure 1. Depletion of gas-phase CH_3Cl by GSH transferases. (A) Individual experiments in which the gas phase was measured at various time points: DM11 (○), GST 5-5 (■), and GST T1 (▲). The control reaction t, devoid of GSH transferase was used to correct for the gas removed for each quantitation. The rates of depletion are estimates based on single-exponential decay (nonlinear regression) fits in the initial region of the curves. The partition ratio was estimated to be 2:1, liquid to gas, based on a comparison of the gas phase in the presence and absence of a blank reaction mix. Each point represents the mean of results from duplicate reactions. (B) Rates of CH_3Cl depletion by each GSH transferase as a function of CH_3Cl concentration.

Dihalomethane Reversion Assays. We previously demonstrated that *S. typhimurium* TA 1535 transformed with a plasmid expressing either rat or human theta class GSH transferases (rat GST 5-5 and human GST T1) yielded a response that was concentration-dependent with respect to the number of revertants and the concentration of dihalomethane (*15*, *31*, *51*, *52*). Six dihalomethanes (CH_2Cl_2, CH_2Br_2, CH_2BrCl, CH_2ICl, CH_2I_2, and CHClF) were examined for their abilities to induce revertants in *S. typhimurium* TA 1535 transformed with plasmids expressing either rat GST 5-5, human GST T1, or bacterial dichloromethane dehalogenase DM11, to characterize the effects of the halogen atoms on biological activity. Levels of enzyme expression at the time of each reversion assay were determined in order to facilitate comparisons.

Results of reversion assays for three of the compounds (CH_2Cl_2, CH_2Br_2, and CH_2BrCl) with bacteria expressing GST 5-5, GST T1, or the control plasmid pKK233-2 are shown in Figure 2 and Table 1. The bacteria expressing GST T1 showed no significant increase in revertants over background for any of the compounds. This result is probably due to very low expression of the enzyme in the

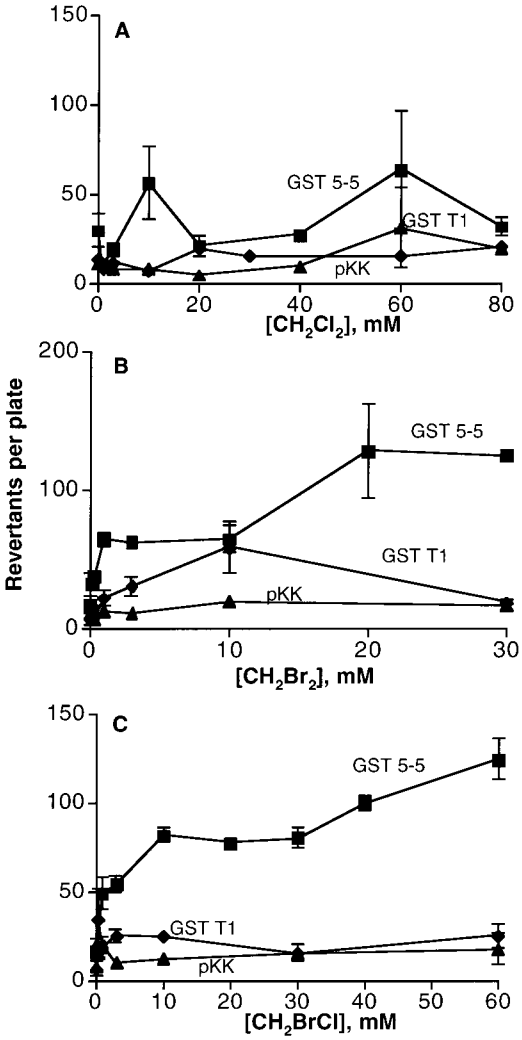


Figure 2. Reversion assays using GSTs 5-5 and T1. *S. typhimurium* TA 1535 transformed with either rat GST 5-5 (■), human GST T1 (◆), or control plasmid pKK233-2 (▼) were incubated with varying concentrations of (A) CH_2Cl_2, (B) CH_2Br_2, or (C) CH_2BrCl for 5 min at 37 °C. Each point represents means of results of duplicate assays. The estimated concentrations of enzymes in liquid culture based on a quantitative immunoblot assay were (respectively) 220, 230, and 230 nM for GST 5-5 and 6, 7, and 7 nM for GST T1 in parts A, B, and C.

Table 1. Mutagenicity of Dihalomethanes Activated by GSH Transferases

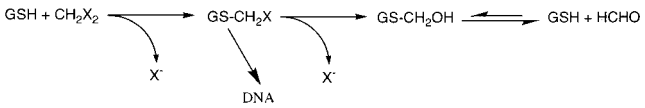
	mutagenicity [revertants mM^{-1} (per plate)][a]			
substrate	DM11[b]	GST 5-5[b]	GST T1	pKK[c]
CH_2I_2	20 ± 29	89 ± 74	<1	<1
CH_2ICl	139 ± 28	177 ± 45	<1	<1
CH_2Br_2	135 ± 47	55 ± 17	5 ± 1	<1
CH_2BrCl	28 ± 9	10 ± 3	<1	<1
CH_2Cl_2	28 ± 5	2 ± 4	<1	<1
CH_2ClF	2 ± 1	2 ± 11	<1	<1

[a] (±) SE from linear regression (per mM dihalomethane). [b] Calculated from linear regression of linear portion of data points from normalized reversion test plots. [c] Vector only.

bacteria (4−7 nM). Similarly, the control group (tester strain transformed with pKK233-2) showed no significant increase in revertants for any of the compounds over the range of concentrations tested.

The bacteria expressing GST 5-5 showed the greatest revertant response with CH_2Br_2 (Figure 2B) and the mixed dihalomethanes CH_2BrCl (Figure 2C) and CH_2ICl (Table 1). However, CH_2Cl_2 (Figure 2A) and CH_2I_2 both produced very low numbers of revertants, and there was only a slight increase as the dihalomethane concentrations were increased (Table 1). Expression of GST 5-5 was estimated to be between 100 and 200 nM in all of the revertant tests, a level much higher than with GST T1.

Scheme 2. Pathway for Conjugation of CH_2X_2 with GSH and Subsequent HCHO Formation



Bacteria expressing DM11 showed a dose-dependent increase in revertants for all of the six dihalomethanes tested (Figure 2). DM11 was expressed at levels between 1 and 2 μM, up to 8-fold greater than GST 5-5. (Interestingly, the DM11 tester strain exhibited a high background rate not seen with either of the mammalian GST tester strains.)[2]

Formation of HCHO by GSH Transferases. The formation of HCHO (Scheme 2) was measured as a function of dihalomethane concentration for six dihalomethanes using the Nash assay, with each of the purified enzymes (Figure 4, Tables 2 and 3). Both of the mammalian theta class enzymes bgin to exhibit saturation at very high concentrations of most of the dihalomethane substrates. A k_{cat} for GST 5-5 could only be estimated with CH_2Br_2 and CH_2I_2 due to the limited solubility of these dihalomethanes (results not shown). The catalytic efficiencies (k_{cat}/K_m) of GST 5-5 and GST T1h (Tables 2 and 3) reveal a similar pattern for the rat and human enzymes. For the dihalomethanes CH_2Cl_2, CH_2Br_2, CH_2ICl, and CH_2BrCl the ratio of GST 5-5 to GST T1h efficiencies ($k_{cat}/K_m^{GST\ 5\text{-}5}$:$k_{cat}/K_m^{GST\ T1}$) was constant at ~3, suggesting that GST T1h is generally 3-fold less efficient in converting dihalomethanes to HCHO.

Comparison of Nash and NAD^+-HCHO Dehydrogenase Assays. Although the Nash assay was suitable for the two mammalian GSH transferases, it was not appropriate for analysis of the DM11 enzyme with most of the dihalomethanes because DM11 has K_m values for these substrates in the micromolar range. At low substrate concentrations, a large fraction of the dihalomethane had to be consumed to generate sufficient levels of product for detection in the Nash assay. To solve this problem, a coupled enzyme assay using HCHO dehydrogenase was used, in which NADH is generated (*45*). The results were compared to the Nash assay to determine if

[2] The reason for this phenomenon is unknown. The background reversion rate is represented by the dotted line in each of the graphs and indicates the true baseline for each compound within the DM11 tester strain. Controls performed with the plasmid system pTrc99A, lacking a gene product, did not show the high background rate, indicating that the plasmid system itself was not the cause of the high background for the DM11 tester strain. The background revertant level was rather variable, from 40 to 300 revertants per plate at various times over the course of 8 months. No difference in the backgound level was seen in this laboratory (Nashville) with DM11 systems prepared separately in Nashville and Zürich. One possibility is that DM11 is activating some medium component that is present at varying levels. To test this possibility, we added the inhibitor $ClCH_2CN$ (*53*) at concentrations of 0−100 μM, which dramatically blocked the activiation of $BrCH_2CH_2Br$ in this system. However, $ClCH_2CN$ had no effect on the background level of revertants. The results indicate that if a contaminating mutagen is present in our system, it is activated by DM11 but not in the typical manner such that the process is inhibited by $ClCH_2CN$.

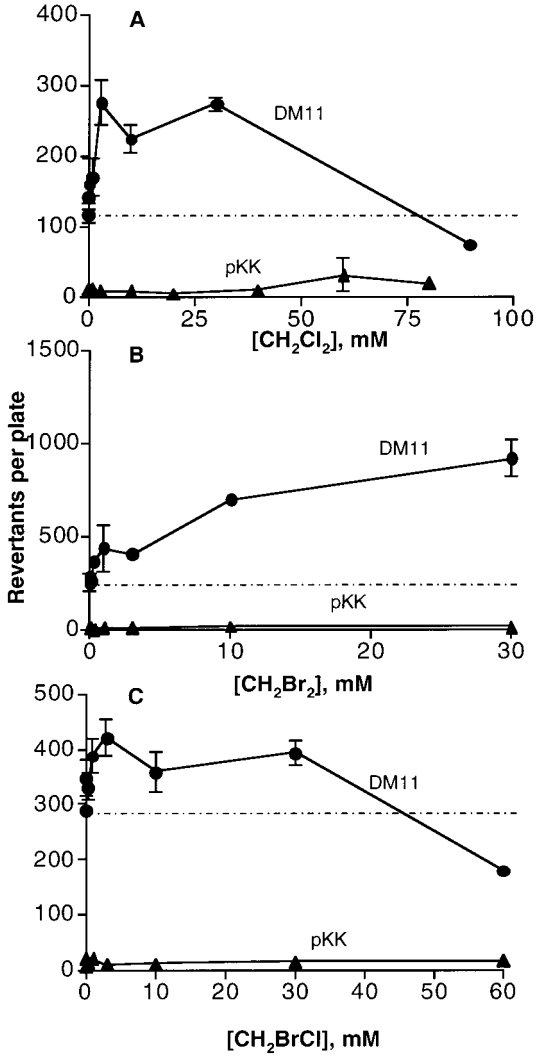


Figure 3. Reversion assays using DM11. *S. typhimurium* TA 1535 transformed with either DM11 (●) or control plasmid pKK233-2 (▲) were incubated with varying concentrations of (A) CH_2Cl_2, (B) CH_2Br_2, or (C) CH_2BrCl for 5 min at 37 °C. Each point represents means of results of duplicate assays. The estimated concentrations of enzyme in the liquid culture based on a quantitative immunoblot assay were 1.7, 1.3, and 1.3 μM in parts A, B, and C, respectively. The dotted line indicates the background rate of reversion for DM11-transformed *S. typhimurium*.

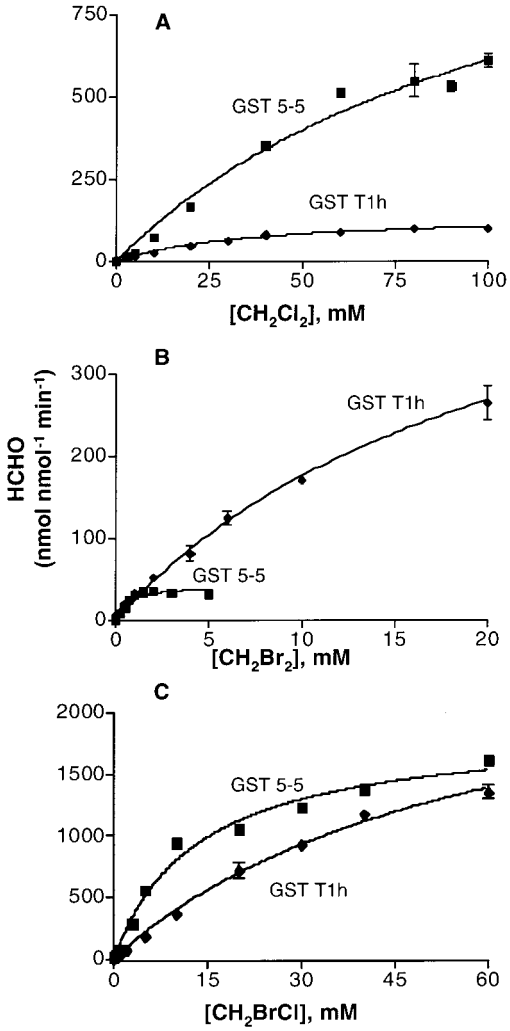


Figure 4. Formation of HCHO from dihalomethanes by GSTs 5-5 and T1 as a function of substrate concentration. Rates of HCHO formation were determined (Nash assay) with GST 5-5 and GST T1 at the indicated concentrations of dihalomethanes. Reactions were carried out at 37 °C for 5 min. Each point represents the mean of duplicate reactions: (A) CH_2Cl_2, (B) CH_2Br_2, (C) CH_2BrCl.

both assays yielded equivalent kinetic constants (Table 4). A comparison of the two methods indicates they provide similar values for K_m and k_{cat} with dihalomethanes having good leaving groups (e.g., Cl, Br, I). With CH_2ClF, the formaldehyde dehydrogenase assay appeared to underestimate k_{cat} and K_m. This result is attributed to the generation of a stable *S*-(1-fluoromethyl)GSH intermediate that does not rapidly decompose to form HCHO at the reaction temperature used. Blocki et al. (*54*) estimated a $t_{1/2}$ of ~30 min at 23 °C. The Nash reaction chemistry requires heat (60 °C, 20 min), which drives the formation of HCHO from the intermediate $GSCH_2F$. The other dihalomethanes tested do not appear to form such stable intermediates, and the HCHO dehydrogenase assay is suitable for following HCHO formation from those compounds.

Formation of HCHO by DM11. DM11 exhibited different kinetic characteristics from the mammalian GSH transferases with dihalomethanes, as well as with monohaloalkanes and 1,2-dihaloethanes (*25*) (Figure 5, Table 5). The DM11 enzyme exhibited K_m values that are several orders of magnitude lower than those observed with GST 5-5 and GST T1h. The catalytic efficiency of DM11 was considerably higher with this group of substrates, due to the relatively low values of K_m. The k_{cat} values varied only 3-fold for DM11, with the exclusion of CH_2ClF, and k_{cat}/K_m values varied only 2-fold when CH_2Cl_2 and CH_2ClF were excluded.

Table 2. Steady-state Kinetic Parameters for GST 5-5 Conjugation of Dihalomethanes

	HCHO formation[a,b]		
substrate	k_{cat} (min^{-1})[d]	K_m (mM)[d]	k_{cat}/K_m (mM^{-1} min^{-1})
CH_2I_2	21 ± 1	0.31 ± 0.05	66 ± 12
CH_2ICl	760 ± 37	4.2 ± 0.6	180 ± 30
CH_2Br_2	42 ± 3	0.53 ± 0.13	78 ± 20
CH_2BrCl	1870 ± 80	13.4 ± 1.6	140 ± 20
CH_2Cl_2	1290 ± 210	112 ± 30	11.5 ± 3.6
CH_2ClF			7.8[c]

[a] All assays were done using the Nash method. [b] (±) SE from nonlinear regression. [c] Value obtained from linear regression. [d] These estimates are not particularly reliable (e.g., Figure 3) and emphasis should be placed on the ratio k_{cat}/K_m.

Table 3. Steady-State Kinetic Parameters for GST T1h Conjugation of Dihalomethanes

	HCHO formation[a,b]		
substrate	k_{cat} (min^{-1})[c]	K_m (mM)[c]	k_{cat}/K_m (mM^{-1} min^{-1})
CH_2I_2	100 ± 10	1.5 ± 0.25	65 ± 13
CH_2ICl	1310 ± 160	23.4 ± 4.4	56 ± 12
CH_2Br_2	562 ± 36	21.9 ± 2.6	26 ± 4
CH_2BrCl	2760 ± 230	59.4 ± 8.0	47 ± 7
CH_2Cl_2	143 ± 7	35.1 ± 4.3	4.1 ± 0.5
CH_2ClF			<4

[a] Nash assay used in all assays. [b] (±) SE from nonlinear regression. [c] These estimates are not particularly reliable (e.g., Figure 3) and more emphasis should be placed on the ratio k_{cat}/K_m.

Table 4. Steady-State Kinetic Parameters for DM11 Comparing Two Analysis Methods

		HCHO formation[a]		
method	substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} min^{-1})
Nash	CH_2Cl_2	150 ± 3	75 ± 15	2.0 ± 0.4
	CH_2ClF	20 ± 1	309 ± 53	0.065 ± 0.012
dehydrogenase	CH_2Cl_2	149 ± 2	45 ± 2	3.3 ± 0.2
	CH_2ClF	3.1 ± 0.2	99 ± 21	0.031 ± 0.007

[a] (±) indicates SE from nonlinear regression.

Table 5. Steady-State Kinetic Parameters for DM11 Conjugation of Dihalomethanes

	HCHO formation[a,b]		
substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} min^{-1})
CH_2I_2	221 ± 10	4.5 ± 1.0	49 ± 11
CH_2I_{Cl}	131 ± 2	1.8 ± 0.3	73 ± 12
CH_2Br_2	412 ± 7	9.6 ± 0.8	43 ± 4
CH_2BrCl	178 ± 4	5.1 ± 0.7	35 ± 5
CH_{2Cl2}	149 ± 2	45 ± 2	3.3 ± 0.2
CH_{2ClF}	3.1 ± 0.2	99 ± 21	0.031 ± 0.007

[a] NAD^+-HCHO dehydrogenase assay used in all assays. [b] (±) SE from nonlinear regression.

Discussion

Efficiency of GSH Transferases Toward Halomethanes. Dihalomethanes cause mutations through activation by GSH transferases (*31*, *47*, *55*−*57*) presumably through the mechanism proposed in Scheme 2. Dihalomethanes are initially conjugated by GSH, in the only step catalyzed by GSH transferases. Thus, the efficiencies of the enzymes are crucial to understanding their roles in the metabolic activation of this class of compounds. The reactive intermediate *S*-halomethylGSH ($GSCH_2X$) formed by the enzyme then reacts with H_2O to form *S*-hydroxymethylGSH, which equilibrates with HCHO and GSH. The likely point of DNA interaction is after the formation of *S*-halomethylGSH (*26*). These compounds have not been isolated due to their presumed lability[3] and similarly DNA adducts formed from the reaction have not been isolated because they are also presumed to be labile (*15*).[4] In these studies, monohalogenated compounds such as CH_3Cl, CH_3CH_2Br, or CH_3CH_2Cl provided a means of preventing postenzymatic chemistry because the immediate product is a stable GSH conjugate rather than a labile *S*-halomethylGSH conjugate. Previously, this technique was used to measure product formation of monohaloethanes (*25*) and was used here to measure substrate depletion of CH_3Cl.

The concentration dependence of the velocity of the DM11 enzyme with CH_3Cl suggests that DM11 follows hyperbolic kinetics whereas GST T1 cannot be saturated with the substrate over the range of concentrations tested (Figure 1B). This pattern also applies for the formation of $GSCH_2CH_3$ from either CH_3CH_2Br or CH_3CH_2Cl (*25*), suggesting that the nonhyperbolic behavior characteristic of GSTs 5-5 and T1 is a function of the enzyme and not a consequence of the assay system, e.g., (gas phase) substrate depletion vs (solution phase) product formation. Thus, the lack of saturation with GSTs 5-5 and T1 and the saturation with DM11 are not related to the presence of a second halide in the substrate. Experiments in which half-mustards were incubated with DM11 or GST 5-5 indicated that decomposition of the *S*-haloethylGSH compounds was not enhanced by the presence of enzyme (*25*). A corollary of this conclusion is that GSH transferases do not appear to be involved in the removal of the second halide from the dihaloalkanes.

The bacterial dichloromethane dehalogenase DM11 is known to be similar to the theta class GSH transferases (26% sequence identity) (*39*) and contains a critical serine residue (Ser12) (*48*) that aligns with Ser10 of rat GST 5-5, suggesting similar catalytic function. Blocki et al. (54) reported that the bacterial dehalogenase does not catalyze conjugation with CH_3Cl, $ClCH_2CH_2Cl$, or CH_3CHCl_2. Results obtained here demonstrated that the DM11 enzyme can conjugate CH_3Cl with GSH in a manner similar to the mammalian theta GSH transferases (Figure 1A). Theta class enzymes have the ability to conjugate epoxides (*49*) with GSH as well as perform dehalogenation reactions (*49*, *60*). In contrast, DM11 does not catalyze the addition of GSH to the theta class substrates 1,2-epoxy-3-(*p*-nitrophenoxy)propane and ethylene oxide (results not shown), in agreement with findings of others (*54*). This fact suggests that the DM11 enzyme has a more restricted substrate selectivity than the mammalian theta GSH transferases. It is reasonable to suppose the narrow substrate selectivity and enhanced catalytic efficiency of the DM11 enzyme toward dihalomethanes is an evolutionary consequence of the conscription of this enzyme to initiate the acquisition of carbon from these materials.

Activation of Dihalomethanes to Mutagens. Mutagenicities calculated from the linear portion of reversion assay plots and normalized for varying protein concentrations are shown in Table 1. The DM11 enzyme induced the greatest reversion rates with CH_2Br_2 and CH_2ICl, and GST 5-5 was also very efficient at enhancing reversion rates with CH_2ICl. This method for comparing reversion rates between compounds did not produce any significant reversion rates for GST T1 for any of the dihalomethanes with the exception of CH_2Br_2 (Table 1). The lack of reversion rates in the presence of GST T1 is probably a combination of low protein expression levels

[3] We have recently determined the $t_{1/2}$ of *S*-(2-acetoxymethyl)GSH as 11 s at pH 8.0 and 23 °C (*58*).

[4] Recently, deoxyribonucleoside adducts have been prepared with the surrogate *S*-(2-acetoxymethyl)GSH (dAdo, dCyd, dThd) and characterized (*15*, *58*). The half-life of the isolated nucleoside adduct *S*-[1-(N^2-deoxyguanosinyl)methyl]GSH appears to be on the order of ~8 h at neutral pH. The dThd adduct was identified following treatment of calf thymus DNA with *S*-(2-acetoxymethyl)GSH. The dCyd adduct is known to be labile (*58*) although the $t_{1/2}$ has not been measured. The stabilities of the adducts may be considerably greater than in nucleosides or bases, e.g., as with some of the etheno adducts (*59*).

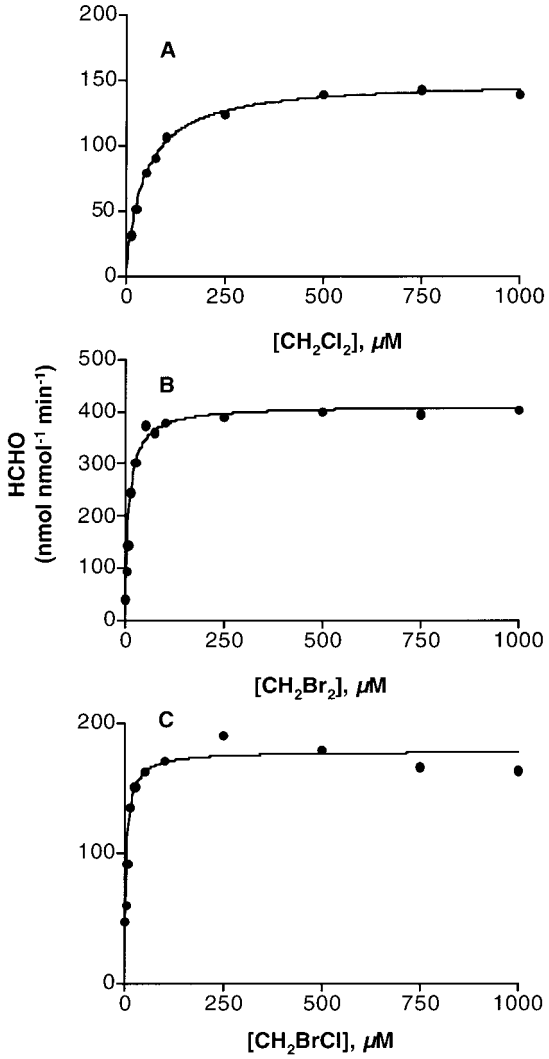


Figure 5. Formation of HCHO from dihalomethanes by DM11 as a function of substrate concentration. Rates of HCHO formation were determined with DM11 at the indicated concentrations of dihalomethanes. Reactions were carried out at 37 °C for 5 min. Each point represents the mean of duplicate reactions. (A) CH_2Cl_2, (B) CH_2Br_2, (C) CH_2BrCl.

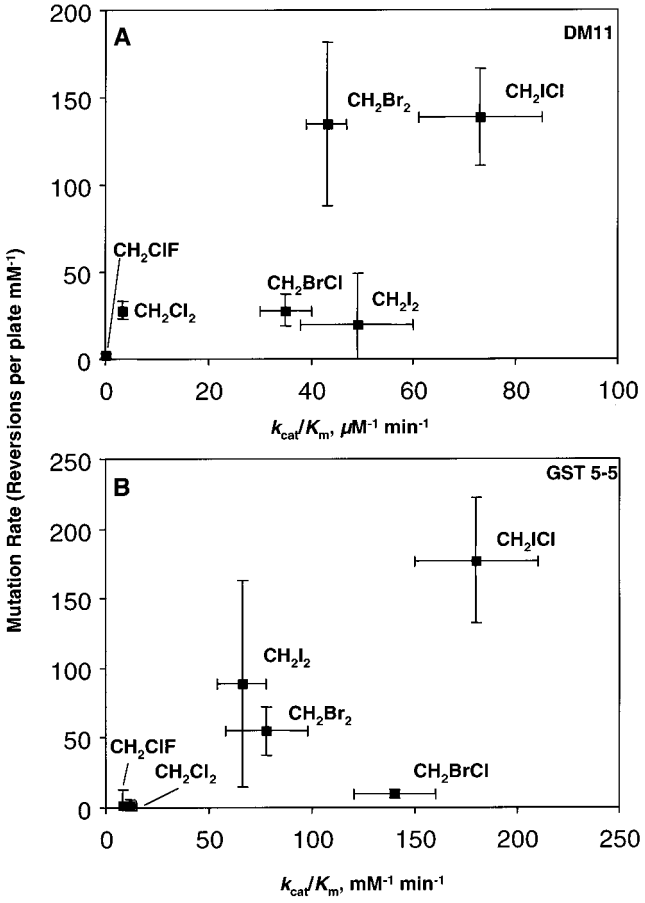


Figure 6. Comparisons of (A) DM11 k_{cat} values for HCHO formation and reversion results. Comparisons of (B) GST 5-5 catalytic efficiencies for HCHO formation and reversion results. Catalytic efficiences and k_{cat} values are plotted against the enzyme concentration (1 μM) normalized reversion test data at for each dihalomethane at 1 mM. (Reversion numbers of <10 were not included in the set).

in *S. typhimurium* and the 3-fold reduced catalytic efficiency of the enzyme toward dihalomethanes.

A significant correlation does not appear to exist between the mutation rates and the catalytic efficiencies of DM11 and GST 5-5 toward dihalomethanes, as illustrated in Figure 6. This result can be taken to suggest that there is little relationship between the formation of HCHO and the observed reversion rates. However, the conclusions that can be drawn from the data are limited, since in many cases the measured reversion rates carried large standard errors. Perhaps more importantly, formation of HCHO is dependent upon the generation of the reactive *S*-halomethylGSH. Even if a relationship did exist between the mutagenicities and HCHO formation, it could be taken to suggest that reversion rates are dependent on the formation of *S*-halomethylGSH and not necessarily HCHO formation.

Heck has proposed that the generation of HCHO is an important component of the rodent carcinogenicity of CH_2Cl_2, due to the potential role of HCHO in producing DNA-protein cross-links (*36*). Although the contribution of such a process to rodent carcinogenicity cannot be completely excluded, research in this and several other laboratories indicates that HCHO is not involved in the GSH transferase-associated mutagenicity (*15*, *29*, *47*). The results presented in Figure 6 provide further evidence that HCHO generation is not the primary cause of the mutations seen in these systems.

Results from reversion tests with *S. typhimurium* TA 1535 expressing DM11 suggest that *S*-halomethylGSH is generated by DM11 during the dehalogenation of dihalomethanes because concentration-dependent increases in revertants were observed (Figure 3). Presumably, DNA adducts are formed in *Methylophilus* in the course of dihalomethane metabolism. Whether mutations accumulate in *Methylophilus* during the course of growth on dihalomethanes is unknown. The maximum reversion rate observed was ~200 revertants for CH_2Cl_2 in DNA repair-deficient *S. typhimurium* TA 1535 (Figure 2). DNA repair mechanisms would reduce this number so that the number of mutations may be small, thereby attenuating any affect of DM11 CH_2Cl_2-induced DNA damage. Expression of rat GST 5-5 in a *Methylobacterium* strain devoid of dehalogenase did not allow growth on CH_2Cl_2, suggesting that the metabolism of CH_2Cl_2 by GST 5-5 yielded a toxic product or the rate of reaction was too slow to remove toxic CH_2Cl_2 (*47*). The possibility exists that DM11 may have some capacity for detoxication of $GSCH_2Cl$, although this suggestion would not seem to be in agreement with the mutagenicity results (Figure 2A).

The mammalian theta class GSH transferases consistently exhibited a nonhyperbolic concentration dependence for a variety of substrates, including monohaloalkanes and 1,2-dihaloethanes (*25*). DM11 does not display this characteristic with any of these substrates. The enzyme efficiently removes low concentrations of dihalomethanes to prevent damage to the bacteria or to generate energy at low substrate concentrations.

Effect of the Leaving Group. One of the goals of this study was to determine the effect of the halogen leaving group in conjugation and mutagenesis for each of the enzymes. These results can be considered first in terms of the assays involving conversion of dihalomethanes to HCHO. In all cases, except with CH_2ClF, the rate-limiting step in formation of HCHO is the initial addition of GSH to the dihalomethane. In the instances where the Nash assay was used the elevated temperature of the derivatization process forces the formation of HCHO from the stable (*54*) $GSCH_2F$ intermediate. Thus, the rate of formation of HCHO should be a reasonable estimate of the rate of the initial addition reaction. The most extensive set of results involved the measurement of enzyme efficiency, k_{cat}/K_m, because of the lack of saturation in the assays with the mammalian GSH transferases. The results were also considered in the context of the halide leaving group abilities observed with monohaloalkanes and 1,2-dihaloethanes (*25*). In those conjugations, all of the three GSH transferases showed a halide order (Br > Cl) of 5−30-fold in k_{cat}/K_m (comparing $BrCH_2CH_2Cl$ to $ClCH_2CH_2Cl$ in the 1,2-dihaloethane series). With all the GSH transferases, a 10−15-fold variation in k_{cat}/K_m was observed among the dihalomethanes, exclusive of the slowly conjugated CH_2ClF (the variation was less when Cl is excluded, i.e., only Br and I are considered as the initial leaving groups). The order seen with GST 5-5 was $CH_2ICl > CH_2BrCl > CH_2Br_2 \approx CH_2I_2 > CH_2Cl_2 \approx CH_2ClF$. A rational explanation for the order may not be obvious, but one possibility is that the electronegativity of the "second" halogen influences the attack of the GSH thiolate on the carbon center ($X_2^{\delta-}-CH_2^{\delta+}-X_1 \rightarrow X_2^{\delta-}-CH_2-SG + X_1$). In this regard (with the I- and Br-containing dihalomethanes), the GST 5-5-catalyzed formation of HCHO was more efficient when the electronegativity of the second halogen increased (Table 2). However, data points for CH_2Cl_2 and CH_2ClF substrate concentration curves overlay directly on each other (results not shown), suggesting that GST 5-5 is treating these substrates as if they were the same.

The order of k_{cat}/K_m for GST T1 was $CH_2I_2 \approx CH_2ICl \approx CH_2BrCl > CH_2Br_2 > CH_2Cl_2 > CH_2ClF$ and for DM11 was $CH_2Br_2 \approx CH_2I_2 > CH_2BrCl > CH_2ICl > CH_2Cl_2 > CH_2ClF$ (Tables 3 and 4). A simple explanation for the pattern does not present itself in these instances. What is consistent about the three GSH transferases is that CH_2Cl_2 and CH_2ClF were least efficiently conjugated. This lower reactivity of Cl relative to Br is consistent with the findings with monohaloalkanes and 1,2-dihaloalkanes (*25*). Other factors such as steric effects, substrate solvation, or the ability of the enzyme to specifically stabilize different leaving groups surely influence the observed catalytic efficiencies and mask influence of the intrinsic leaving group abilities on the recations.

One of the difficulties in the interpretation of the work with the mammalian GSH transferases is the difficulty in determining both k_{cat} and k_{cat}/K_m because of the lack of saturability with all of the substrates, including monohaloalkanes and 1,2-dihaloalkanes (*25*). In contrast, the DM11 enzyme does follow saturation kinetics so that k_{cat} can be estimated accurately (Figure 4). Interestingly, the conjugations of CH_3CH_2Br and CH_3CH_2Cl both had the same k_{cat} (45 min^{-1}) (*25*) and the Br/Cl difference was only 3-fold for the 1,2-dihaloethanes ($BrCH_2CH_2Cl$ vs $ClCH_2CH_2Cl$). Among the dihalomethanes the order $CH_2Br_2 > CH_2I_2 > CH_2Cl_2 > CH_2BrCl \approx CH_2ICl > CH_2ClF$ was seen for k_{cat}, but the difference between CH_2Br_2 and CH_2ICl was only 4-fold. The highest k_{cat} (CH_2Br_2, 240 min^{-1}) was considerably more than that measured for CH_3CH_2Br or CH_3CH_2Cl (45 min^{-1}) (*25*). These results indicate that a step other than displacement of the halogen from the thiolate reaction complex is rate-limiting, because of the narrow range of k_{cat}. In addition, an influence of nonproductive binding on the observed k_{cat} values cannot be excluded.

It is of interest to note that GST 5-5 was about five times× more active than GST T1. This apparent difference in intrinsic catalytic activity (at least the catalytic activities considered here and in ref *25*) must be considered in the context of any potential differences produced by the substitutions used in the recombinant enzymes (i.e., pentahistidine) and in the levels of expression of the enzymes in tissues.

Conclusions. The bacterial DM11 enzyme exhibits a much higher catalytic efficiency and greater ability to induce mutations with dihalomethanes than its mammalian counterparts. The mutagenicity studies clearly establish that HCHO cannot explain the base-pair mutations, even if HCHO is generated inside the cells. Thus it is likely that the mutation rates with various dihalomethanes are a complex function of the efficiency of generation and the lifetime of reactive *S*-halomethylglutathione intermediates. If the $GSCH_2X$ intermediates are as unstable as the model compounds indicate, diffusion within the cell may be a crucial factor in the observed mutagenesis.[5] We have recently estimated the $t_{1/2}$ of $GSCH_2OAc$ as 11 s (*58*), so it may provide a better opportunity to study some of these reactions. Moreover, it may be of interest to further evaluate CH_2ClF in light of the reported stability of its conjugate (*54*) and the apparently high mutagenicity to k_{cat}/K_m ratio in Figure 6.

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Supporting Information Available: Figure for determination of the $K_{m,GSH}$ and k_{cat} for rat GST 5-5, used for this manuscript and in the previous paper in this issue regarding mono and 1,2-dihaloethanes (*25*). This material is available free of charge via the Internet at http://pubs.acs.org.

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