Activation of Dihaloalkanes by Thiol-dependent Mechanisms

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Dihaloalkanes constitute an important group of chemicals because of their widespread use in industry and agriculture and their potential for causing toxicity and cancer. Chronic toxic effects are considered to depend upon bioactivation, either by oxidation or thiol conjugation. Considerable evidence links genotoxicity and cancer with glutathione conjugations reactions, and some aspects of the mechanisms have been clarified with 1,2-dihaloalkanes and dihalomethanes. Recently the DNA repair protein \(O^6\)-alkylguanine transferase has been shown to produce cytotoxicity and genotoxicity by means of a thiol-dependent process with similarities to the glutathione reactions.

Keywords: \(O^6\)-Alkylguanine transferase, Bioactivation, Dihalomethanes, Ethylene dibromide, Glutathione, Mutations

Introduction

Halogenated chemicals have many uses in industrial processes, particularly as solvents. Dihalogenated alkanes are commonly used as solvents (e.g. \(\text{CH}_2\text{Cl}_2\)) and have also been used as precursors of vinyl monomers (e.g. \(\text{CICH}_2\text{CH}_2\text{Cl}\)), pesticides (\(\text{BrCH}_2\text{CH}_2\text{Br}\)), gasoline additives (\(\text{BrCH}_2\text{CH}_2\text{Br}\)), and synthetic building blocks. The toxicity of dihaloalkanes requires some special considerations. Industrial workers have the potential to be exposed to large amounts of some of these solvents in accidents, and deaths have resulted from acute toxicity (Letz et al., 1984). Another issue is the risk of cancer from long-term chronic exposure. For instance, \(\text{BrCH}_2\text{CH}_2\text{Br}\) was withdrawn from industrial use because of these concerns (Sun, 1984) and the potential of \(\text{CH}_2\text{Cl}_2\) for causing human cancer is an ongoing problem in risk assessment (Andersen et al., 1987; Reitz et al., 1989).

All halogenated hydrocarbons can be acutely toxic at high doses due to their general anesthetic properties. However, genotoxicity considerations relevant to cancer almost certainly involve bioactivation processes. Dihaloalkanes are bifunctional electrophiles and their activation shows some unusual properties. Conjugation of chemicals with the tripeptide glutathione (GSH) is usually a detoxication process in the body (Armstrong, 1997). However, most dihaloalkanes can be activated by either oxidation (cytochrome P450 (P450)) or by GSH conjugation by GSH transferases, e.g. \(\text{BrCH}_2\text{CH}_2\text{Br}\) (Fig. 1). Subsequently the chemistry involved in the GSH pathway has been extended to the DNA repair protein \(O^6\)-alkylguanine transferase (AGT).

Ethylene Dibromide: Characterization of the Pathway to DNA Adducts

Early work from the laboratories of Rannug (Rannug et al., 1978; Rannug and Beije 1979) and Breimer (van Bladeren et al., 1979) indicated that the genotoxicity of ethylene dibromides was dependent upon cytosolic enzymes and not microsomal oxidation (e.g. P450). These basic results, repeated in our own laboratory (Guengerich et al., 1980), suggested that a GSH conjugation pathway such as that shown in Fig. 1 might be operative. Subsequently we were able to demonstrate the incorporation of radiolabel from both \(\text{BrCH}_2\text{CH}_2\text{Br}\) and GSH into DNA, either with GSH transferase or in rat hepatocytes (Ozawa and Guengerich, 1983). The major DNA adduct formed was demonstrated to be the \(N^7\)-guanyl adduct shown in Fig. 1, first by Raney Ni reduction to \(N^7\)-ethylguanine (Ozawa and Guengerich, 1983) and subsequently by NMR and mass spectrometry (Koga et al., 1986). This adduct was found to be the major one formed in liver or kidney DNA of rats treated with \(\text{BrCH}_2\text{CH}_2\text{Br}\) (Inskeep et al., 1986).

Further work indicated that GSH transferases yield the highest amount of GSH-alkyl-DNA adducts (in vitro) with \(\text{BrCH}_2\text{CH}_2\text{Br}\) compared to 1,3-dibromopropane and 1,4-dibromobutane (Inskeep and Guengerich, 1984). These results suggested the possibility of anchimeric assistance in the
mechanism of reaction of a GSH half-mustard with DNA. The
mechanism was addressed in a series of experiments involving
kinetics and stereochemistry (Peterson et al., 1988). In the
kinetic analysis, the sulfur atom was found to be prerequisite
for reaction, arguing for its role in activation and against a
direct displacement (of the halide). Further, a free amino
group was not required for reaction in a series of homologs of
related cysteine half-mustards. A stereochemical experiment
is outlined in Fig. 2. NMR analysis of the DNA adduct is only
consistent with an odd number of SN2-type inversions (e.g.,
three in this case) and thus supports the scheme outlined in
Fig. 2 (Peterson et al., 1988).

Some minor DNA adducts have also been characterized,
being generated from BrCH2CH2Br or GSCH2CH2Cl in rat liver
or DNA, respectively. These include S-[2-(N'-adenyl)ethyl] GSH (Kim et al., 1990) and S-[2-(N'-guanyl)ethyl]GSH and S-
[2-(O'-guanyl)ethyl]GSH (Cmarik et al., 1992). Synthetic N'-
adenyl and N'-cytidinyl adducts were prepared and used in
analyses but neither of these adducts were detected in DNA
treated with GSCH2CH2Cl (Cmarik et al., 1992).

Processing of Ethylene Dibromide-GSH Adducts

The S-[2-(N'-guanyl)ethyl]GSH adduct (Fig. 1) has a labile
glycosidic bond and undergoes non-enzymatic depurination,
with a half-life of 70-100 h in various rat tissues (Inskeep et
al., 1986). The adducted guanine is processed by the usual
GSH degradation pathway to yield the mercapturic acid S-
(2-(N'-guanyl)ethyl]-N-acetylcysteine, which is excreted in rat
urine (Fig. 3) (Kim and Guengerich, 1989). The DNA N'-
guanyl adduct did not readily undergo ring-opening of the
imidazole ring to a formamidopyrimidine derivative under
physiological conditions (Cmarik et al., 1992).

The addition of GSH half-mustards (GSCH2CH2X, where
X = halogen) to bacteria yielded DNA adducts and mutations
(Humphreys et al., 1990). Considerations of the levels of
DNA adducts and mutation frequency suggested that the
N'-guanyl adduct could explain the mutagenicity. In other
experiments in which bacteriophage DNA was treated with
GSCH2CH2Cl and then replicated in bacteria, the mutation
spectrum was found to be dominated by GC to AT transitions
(Cmarik et al., 1992). This signature argues strongly against a
role for abasic sites (generated by depurination) as the basis of
the mutations, in that a G → T transversion spectrum would
have been expected (Sagher and Strauss, 1983).

Oligonucleotides corresponding to a sequence of the
bacteriophage DNA prone to mutation (Cmarik et al., 1992)
were prepared with each of the three known GSH-ethylene
conjugates that have been found with BrCH2CH2Br systems,
i.e. the N'-, N'-, and O'-guanyl derivatives (Kim and
Guengerich, 1997). In vivo experiments have not been done,
but in vitro work has shown that all three of these adducts
have the capability of blocking Escherichia coli polymerases
(I and II) and causing at least some misincorporation (Kim
and Guengerich, 1998). A more precise accounting of the
contribution of each adduct to the overall mutagenicity in a
cellular system is not yet available. However, it is of interest to
note that recent experiments with an E. coli his reversion
assay indicate that the expression of the DNA repair protein
AGT (shown to act on the S-[2-(O'-guanyl)ethyl]GSH adduct)
can partially reduce the extent of bacterial mutation (Liu et al.,
2002). Studies with other bacterial systems, Drosophila, and
mammalian systems have also shown dominant G → A
mutation spectra with BrCH$_2$CH$_2$Br (Foster et al., 1988; Ballering et al., 1994; Fossett et al., 1995; Graves et al., 1995). Recently we have used a yeast-based p53 mutation system, based on an ade reporter (Moshinsky and Wogan, 1997), to evaluate the mutation spectrum generated by GSCH$_2$CH$_2$Cl. As with lacI (Cmarik et al., 1992) and the other systems, a strong G $\rightarrow$ A pattern dominated (Valadez, J. G., and Guengerich, F. P., in preparation). One of the concerns in our earlier work (Cmarik et al., 1992) was the limited overlap between the mutation spectrum and the pattern of DNA damage (examined in an isolated oligonucleotide). With the p53 system, we were able to utilize a fluorescence-based ligation-mediated PCR method to analyze the patterns of DNA damage within the yeast at varying times after treatment. After 12 h the residual DNA damage (piperidine-cleaved sites) showed a good match with the mutation spectrum. These results are interpreted to mean that the sequence selectivity of DNA repair (probably nucleotide excision repair) is a dominant factor in generating the observed mutation spectrum, at least in this system.

**DNA Adducts Formed with Other Dihaloalkanes (> 1-Carbon)**

In early work with the GSH-dependent activation, we reported evidence for activation of DBCP and tris-(2,3-dibromopropyl) phosphate, as well as BrCH$_2$CH$_2$Br (Inkeep and Guengerich, 1984). Subsequently we found a far lower level of DNA adduct formation in rat liver with CICH$_2$CH$_2$Cl than BrCH$_2$CH$_2$Br (Inkeep et al., 1986). This halide order also holds in the activation of these dihaloethanes to mutagens in bacteria expressing mammalian GSH transferases (Wheeler et al., 2001a). Apparently the rate of conjugation to generate the initial half-mustard is an issue. DBCP reacts with GSH to generate a series of DNA adducts (Humphreys et al., 1991) (Fig. 4). These adducts are complex because of the potential trifunctional alkylation capability of this compound. Reaction of a DBCP-GSH conjugate with calf thymus DNA yielded the N'-guanyl adducts plus the intra-strand crosslinked guanines shown in Fig. 4. We have not further evaluated the capability of the reagent to form interstrand crosslinks.

We can summarize the work in this area by stating that most 1,2-bifunctional alkyls are capable of causing genotoxic damage by this GSH-dependent pathway, with the leaving group order playing a major role in the extent of binding and biological activities (Thier et al., 1996; Wheeler et al., 2001b).

**Dihalomethanes**

The major pathways of metabolism of dihalomethanes were elucidated by Anders’ group (Ahmed and Anders, 1976; Ahmed et al., 1977) and parallel those subsequently developed for dihaloethanes (Figs. 1, 5). The carcinogenicity of CH$_2$Cl$_2$ in mouse liver and lung has raised issues regarding risk assessment in regard to this commodity solvent (Anderson and Maronpot, 1993; Huff et al., 1996). Rats and hamsters are far less likely to develop tumors, and the question of the proper model for humans has been raised (Graves et al., 1995; Rhomberg, 1995).

The lack of saturability of tumor yield (with respect to dose of CH$_2$Cl$_2$) in mice had been suggested to imply that the GSH transferase-catalyzed conjugation is involved in CH$_2$Cl$_2$ tumorigenicity (Andersen et al., 1987; Reitz et al., 1989). However, experimental attempts to demonstrate CH$_2$Cl$_2$ bioactivation had been relatively inconclusive (van Bladeren et al., 1980; Green, 1983). Our laboratory, in collaboration with Ketterer’s group, succeeded in expressing the rat theta class GSH transferase in the bacterial tester strain Salmonella typhimurium TA1535 (Their et al., 1993). Addition of BrCH$_2$CH$_2$Br, CH$_2$Br$_2$, CHBrCl, or CH$_2$Cl$_2$ to the bacteria yielded base pair mutations in the absence of any additional activation system (Fig. 6).

The conjugation of dihalomethanes with GSH yields formaldehyde (Fig. 5). However, formaldehyde was not mutagenic when added to this system (Thier et al., 1993). A series of studies with several GSH transferases and dihalomethanes shows a lack of correlation between formaldehyde production and mutagenicity, further arguing for another species, i.e. presumably a GSH conjugate (Wheeler et al., 2001b). The half-lives of GSCH$_2$X conjugates are probably considerably less than of GSCH$_2$CH$_2$X homologs. GSCH$_2$CH$_2$OAc is quite stable (Wheeler et al., 2001a) while GSCH$_2$OAc has a half-life of ~12 s (Marsch et
The work of Dekant and Anders (Hashmi et al., 1994) also suggests a short half-life for GSCH2Cl. Presumably DNA adducts are formed from GSCH2X compounds, in order to explain the bacterial mutagenicity (Fig. 6). Vuilleumier used a bacterial GSH transferase to demonstrate the covalent binding of label from both CH2Cl2 and GSH to DNA (Kayser and Vuilleumier, 2001). Characterization of individual nucleoside and DNA adducts has been challenging because of the instability. We reacted synthetic GSCH2OAc, a model for the GSH conjugate, with individual nucleosides and characterized four adducts (Fig. 7) (Their et al., 1993; Marsch et al., 2001). None of these are particularly stable, and we have spent considerable effort in developing rapid methods of DNA digestion and HPLC/mass spectrometry to analyze these. At this time we have tentative evidence that all of these can be formed in DNA treated with GSCH2OAc and that the guanine and thymidine adducts can be detected in DNA incubated with a bacterial GSH transferase, CH2Br2, and GSH (Marsch, G. A. and Guengerich, F. P., unpublished results).

The work has been extended to trihalomethanes by Pegram and his associates (DeMarini et al., 1997; Pegram et al., 1997). Although no DNA adducts have been characterized as of yet, the mechanism appears to parallel that shown for dihalomethanes. GSH conjugation yields formic acid and produces mutations in GSH transferase-expressing S. typhimurium tester strains, suggesting a mechanism analogous to that shown in Fig. 5.

**Activation of Dihaloalkanes by a DNA Repair Protein**

AGT is an enzyme found in organisms from bacteria to humans and has an important role in DNA repair, removing alkyl groups from the O6 atom of guanine to regenerate the normal base (Karran et al., 1979). However, several reports have appeared indicating that the expression of AGT (bacterial, rodent, or human) in E. coli increases the cytotoxicity and mutagenicity of BrCH2CH2Br and CH2Br2 in bacteria (Foster et al., 1988; Abril et al., 1995; Abril et al., 1997; Abril and Margison, 1999). The relationship with the previously documented GSH pathway was not clear from work with bacterial strains devoid of GSH or the uvrABC repair system (Foster et al., 1988; Abril et al., 1995). In our preliminary collaborative work with the Pegg group, we found that expression of AGT in E. coli enhanced the mutagenicity of BrCH2CH2Br but decreased the mutagenicity of added GSCH2CH2Cl (Liu et al., 2002). The latter results are consistent with an at least partial role in mutagenesis for the S-[N\textsuperscript{2}-(O\textsuperscript{6}-guanyl)ethyl]GSH adduct (Cmarik et al., 1992; Kim and Guengerich, 1998), which was shown to be a substrate for AGT (Liu et al., 2002).
The enhancement of cytotoxicity and mutagenicity of dihaloalkanes by a DNA repair protein was an unusual finding, and a mechanism was not immediately obvious. Some of the possibilities that can be considered are shown in Fig. 8. In Part A, DNA reacts with BrCH₂CH₂Br to form an adduct “X”, which then interacts with AGT to form either another adduct (“Y”) or crosslinks AGT to the DNA. In Part B a crosslink (or a toxic product “Y”) is formed following the initial reaction of BrCH₂CH₂Br (DBE) with AGT. Other possibilities can be considered, such as the sequestration of AGT at a DNA-BrCH₂CH₂Br lesion to prevent other modes of repair.

The current evidence favors Pathway B of Fig. 8. Reaction of AGT with BrCH₂CH₂Br inhibited its subsequent activity in removing guanyl O⁶-methyl groups, and the covalent addition of the active site cysteine (Cys145) of AGT could be demonstrated using radioactive labeling and mass spectrometry (Liu et al., 2002). Further, the substitution C145A abolished the mutagenic response. Subsequent work showed the crosslinking of AGT to oligonucleotides in the presence of BrCH₂CH₂Br (Liu et al., 2002).

Similar mechanisms appear to be operative in the case of CH₂Br₂. Reaction of BrCH₂OAc with AGT yielded a mass spectrum corresponding to the addition of –CH₂OAc, presumably to the active site Cys145 because the C145S and C145A proteins yielded negative results.

Conclusions

A general theme of this work has been that thiol-dependent systems that normally have protective roles can be involved in the bioactivation of dihaloethanes and other bifunctional electrophiles (Oda et al., 1996; Their et al., 1996). The detrimental bioactivation is probably related to non-selectivity resulting from the low pKₐ values of GSH (due to GSH transferase) and AGT (both ~6.6) (Graminski et al., 1989) (Guengerich, F. P., and Pegg, A. E., unpublished). A current summary of the activation of BrCH₂CH₂Br by the GSH pathway is presented in Fig. 9A, with potential genotoxicity of

Fig. 9. Summary of postulated mechanisms of genotoxicity of EDB. A, GSH conjugation (Cmarik et al., 1992; Kim and Guengerich, 1998). B, AGT conjugation and DNA crosslinking (Liu et al., 2002).
all three known guanine adducts (although the exact basis of the dominant G → A transition remains unknown). The AGT pathway (Fig. 9B) has considerable chemical similarity, at least in the early phases. The crosslinking of AGT to DNA can readily explain cytotoxicity, due to blockage of DNA polymerase. The mechanism of mutagenicity is less clear. One possibility is that, as with the N7-guanylyl adduct of the GSH pathway, depurination occurs and yields mutations. If this hypothesis is valid, AGT-dependent BrCH2CH2Br mu
genesis should yield a mutation spectrum dominated by G to T transversions (Sagher and Strauss, 1983), distinct from the G to A pattern seen in the GSH pathway (Part A) (Cmarik et al., 1992). However, a more complex pathway involving some error-prone repair process cannot be ruled out with the evidence currently available (Fig. 9B).

The above paradigms have also been generally applicable to dihalomethanes (Fig. 5), although the chemistry of reactions with the GSH conjugates differs considerably in its lack of S2 character and yields very different DNA adducts than BrCH2CH2Br (Fig. 7). Several other bifunctional electrophiles also show enhancement of genotoxicity as the result of GSH conjugation, although the mechanisms are not well-established (e.g., butadiene diepoxide) (Their et al., 1995, Their et al., 1996).

Finally, the demonstration of both GSH transferase and AGT pathways of activation of dihaloethanes and dihalomethanes has been done largely in bacterial model systems because of the complexity of the mechanisms. However, the issue of which of these predominate in relevant mammalian systems must ultimately be addressed, i.e. under conditions where one or the other system is not artificially expressed or attenuated. In the future sensitive mass spectrometry assays and perhaps mutation spectra will be useful in such work.


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