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Mass spectrometry of metallothionein adducts as candidate biomarkers of styrene oxide and 1-phenylpropylene oxide

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**Mass Spectrometry of Metallothionein Adducts as Candidate Biomarkers of
Styrene Oxide and 1-Phenylpropylene Oxide**

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A Thesis
Submitted to
The School of Pharmacy
At
West Virginia University

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For the degree of

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In
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ABSTRACT

Mass Spectrometry of Metallothionein Adducts as Candidate Biomarkers of Styrene Oxide and 1-Phenylpropylene Oxide

Sandra G. Tarr

Metallothioneins (MT) are cysteine-rich intracellular proteins that sequester epoxides, such as the genotoxic styrene metabolite, styrene oxide (SO). Covalent adduct formation by the ring-opening of SO and its homologue, 1-phenylpropylene oxide (*trans*), with MT forms stable complexes that potentially may serve as biomarkers for epoxide exposure. MT was reacted with SO and *trans*-phenylpropylene oxide (PPO) separately in water. Mass spectra of adducts were obtained using electrospray ionization with positive-ion detection in an ion trap mass spectrometer. MS/MS analysis established the covalent nature of the complexation. Further experiments were conducted to determine the site of adduct formation. Molecular modeling was used to predict the most likely site of adduct formation. Alkylation and digestion were used to locate the peptide fragment where adduct formation occurred and confirm modeling results. The likeliest cysteine residue was residue 48 in the alpha domain of MT. MT-SO adducts provide a basis for the development of biomarkers.

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LIST OF ABBREVIATIONS

apo-MT	apo-metallothionein
DTT	dithiothreitol
ESI	electrospray ionization
EDTA	ethylenediaminetetraacetic acid
MS/MS	tandem mass spectrometry
MT	metallothionein
m/z	mass to charge
PPO	<i>trans</i> -phenylpropylene oxide
SO	styrene oxide
TFA	trifluoroacetic acid

CHAPTER I

1. Introduction

Metallothioneins are small intracellular cysteine-rich proteins 60-62 amino acid residues in size. Metallothioneins have been found in practically all life forms and have been isolated from different organs such as the liver, kidney, and brain (Sanz-Nebot, 2003). In mammals, the two major isoforms, MT1 and MT2, are the most abundant in the liver, kidney, pancreas, and intestines (Zangger, 2001).

The conventional nomenclature division of MT1 versus MT2 refers to the absence (MT1) or presence (MT2) of an acidic amino acid residue in position 10 or 11 of the protein sequence (Sanz-Nebot, 2003). Isoforms with minor differences are detected as subgroups of the two main isoforms (MT1 and MT2). The subgroup's specific biological roles still remain unknown.

Mammalian metallothionein binds 7 divalent metal ions to its 20 cysteine residues (Hong, 2001). Metallothionein is comprised of two globular domains. The N-terminal β -domain, residues 1-30, binds three metals to nine cysteine residues in a cluster formation. The C-terminal α -domain, residues 31-61, binds four metals to eleven cysteine residues in another cluster formation (Zangger, 2001).

Metallothioneins are believed to play a physiological role in the metabolism of toxic metals including zinc, copper and cadmium (Gehrig, 1999). Metallothionein has also been shown to be involved in cellular defense mechanisms associated with oxidation and stress (Koh, 2001). Overexpression of metallothioneins is readily induced by a variety of endogenous and exogenous chemicals and elevated metallothionein levels have been correlated with resistance to electrophilic molecules used in chemotherapy (He, 2000). The proposed mechanism is that increased amounts of metallothionein provide increased intracellular drug sequestration, thus reducing the amount reaching the target DNA (Zaia, 1996).

Metallothionein has been previously shown to form covalent adducts with various therapeutic agents. These include chlorambucil (Zaia, 1996), the anticancer agent melphalan (Yu, 1995), the nitrogen mustard mechlorethamine (Antoine, 1998), and phosphoramidate mustard (Wei, 1999).

Styrene is a widely used industrial chemical. Chronic exposure to styrene has been shown to produce reproductive, developmental, genotoxic, and carcinogenic effects in laboratory animals (Linhart, 2001). Styrene's main metabolite, styrene oxide (SO), is considered to be responsible for the toxic effects of exposure to styrene. The epoxide is formed from styrene through microsomal monooxygenase (Watabe, 1981).

Smoking of methamphetamine hydrochloride laced tobacco yields several volatile products formed through pyrolysis. One such product is 1-phenylpropene (Sato, 2001). *trans*-phenylpropylene oxide (PPO) is a metabolite of 1-phenylpropene as well as a homologue of styrene oxide. The objective of our experiments was to form adducts between styrene oxide and *trans*-phenylpropylene oxide with metallothionein.

Various analytical techniques have been used to characterize metallothionein. Isoelectric focusing has been used to separate isoforms of rabbit metallothionein (Nordberg, 1972). Matrix-assisted laser desorption ionization (MALDI) mass spectrometry has been used to study the interaction of sulfur mustard exposure with metallothionein (Price, 2000). Metallothionein dimers have been characterized by nano-spray mass spectrometry (Hathout, 2002). Liquid chromatography and capillary electrophoresis are also techniques employed to characterize metallothioneins (Dabrio, 2002).

The analytical technique used to characterize metallothionein adduct formation was mass spectrometry. Mass spectrometry affords a sensitive and selective mode of analysis. In particular, electrospray ionization mass spectrometry (ESI) was utilized for these experiments. Electrospray ionization is very useful for samples containing large, non-volatile molecules such as proteins (Prange, 2002).

For electrospray ionization, a solution containing the sample and electrolytes is pumped through a metal capillary needle that has a high potential applied to it. The electric field at the tip of the capillary needle generates an aerosol of charged droplets. These droplets are attracted to an orifice in the interface that carries an opposite charge from that of the capillary needle. As the droplets flow toward the orifice, the droplets desolvate, leaving behind charged ions.

Electrospray ionization is a soft ionization technique that keeps the molecular structure of the proteins intact. Thus, molecular and structural information can be obtained.

Once adduct formation is established, the next step is to locate the site of adduct formation. This can be done in a variety of ways. One approach is to use molecular modeling to predict where adduct formation occurs. The modeling predictions are then confirmed experimentally. For example, the protein could be digested and sequenced to verify the modeling results.

Molecular modeling has become an important and powerful tool in understanding the structure of proteins. Molecular structure conformations are simulated using a molecular mechanics approach with force field based calculations. The force field represents energy calculations that contain information such as bond lengths, bond angles, and torsion angles. Reactions can be modeled *in silico* to view predicted information on atomic motions that can not be readily observed experimentally. Comparison to experimental data, where available, is then done to confirm modeling predictions. Structural studies of metallothionein have been done using molecular modeling (Rigby, 2004), and modeling has also been used to study drug interactions with metallothionein (Szilagyi, 2000).

As previously stated, the objective of these experiments was to form covalent adducts between styrene oxide and *trans*-phenylpropylene oxide with metallothionein. The formation of adducts between metallothionein and styrene oxide or *trans*-phenylpropylene oxide could further demonstrate the function of metallothionein in the body. Metallothionein-styrene oxide adducts may also provide a basis for the development of biomarkers for epoxide exposure.

2. Methods and Materials

Chemicals and Reagents. Metallothionein-I from rabbit liver was purchased from Sigma Chemical. The metal content of Metallothionein-I from rabbit liver is approximately 7% of cadmium and zinc. Metallothionein-I is essentially salt free. Styrene oxide and *trans*-phenylpropylene oxide were purchased from Aldrich Chemical. Dithiothreitol, guanidine hydrochloride, and Tris buffer were purchased from Sigma Chemical. Trifluoroacetic acid and ethylenediaminetetraacetic acid (EDTA) were purchased from Fisher Chemical. Acetonitrile and methanol were HPLC grade and were purchased from Fisher Chemical. Methyl 4-nitrobenzenesulfonate was purchased from Aldrich Chemical.

The one milliliter C18 solid phase extraction cartridges were purchased from Varian Corporation. The PD-10 desalting columns were purchased from Amersham Biosciences.

Metal Removal Process. apo-Metallothionein was obtained through a treatment process of the purchased metallothionein-I based on a literature method (Zaia, 1998). apo-MT-I was prepared by adding trifluoroacetic acid solution (0.02%) to MT-I in water (100 μ g, 16 μ M). The sample was thoroughly mixed and allowed to sit at room temperature for one-half hour. The metals were removed by solid phase extraction. A one milliliter C-18 extraction cartridge was first conditioned by allowing two one-milliliter portions of methanol to flow through the cartridge, followed by two one-milliliter portions of water. The sample was loaded onto the cartridge and allowed to flow completely through. The cartridge was then washed with two two-hundred microliter washes of 0.1% trifluoroacetic acid. The sample was eluted from the cartridge by one two-hundred microliter elution of an acetonitrile: water (30:70) mixture.

Adduct Formation. The procedure for forming adducts between metallothionein and styrene oxide was a simple mixture step based on a method for forming glutathione-styrene oxide adducts (Ishida, 1989). Styrene oxide (500 μ g, 4.2 mM) was added to a MT-I sample (100 μ g, 16 μ M). The sample was rocked for one-half hour at room temperature. Following the mixing step, the samples were taken through the metal

removal process. Twenty microliters of 10 mM ammonium acetate was added to the sample and mixed before infusion into the mass spectrometer.

The method for forming adducts between apo-metallothionein and styrene oxide was similar to the procedure for metallothionein. Styrene oxide (500 μ g, 4.2 mM) was added to the apo-metallothionein (100 μ g, 16 μ M) sample and mixed for one-half hour at room temperature. In a test to increase adduct formation, DTT (0.046 mg, 0.3 mM) was added to a portion of the samples. Twenty microliters of 10mM ammonium acetate was added to the final sample and mixed before infusion into the mass spectrometer.

The method for forming adducts between metallothionein and *trans*-phenylpropylene oxide was a simple mixture step. *trans*-phenylpropylene oxide (0.04 mg, 0.3 mM) was added to the metallothionein sample (100 μ g, 16 μ M). The sample was rocked for one-half hour at room temperature and was allowed to stand for 24 additional hours at room temperature. The samples were then taken through the metal removal process. Twenty microliters of 10 mM ammonium acetate was added to the sample and mixed before infusion into the mass spectrometer.

Mass Spectral Analysis of Adduct Formation. A Finnigan Deca LCQ quadrupole ion trap mass spectrometer was used for adduct sample analysis. The source utilized electrospray ionization. Ions were monitored in the positive ionization mode. The mass analyzer was an ion trap. The samples were infused into the mass spectrometer at a flow rate of 7 μ L/min. The heated capillary temperature was 160 °C. The spray voltage was set to 5.21 kV, and the capillary voltage was at 50 V. The sheath gas flow rate was 39 mL/min. MS/MS conditions were 2 amu range on the parent mass and the collision energy range was from 30-75%.

Molecular Modeling. For the modeling of the styrene oxide adduct, a search was first executed at the RCSB Protein Data Bank and only rabbit metallothionein-2 (alpha and beta domain) was found. Next a search was done at the Swiss Prot site for rabbit metallothionein-1. Rabbit metallothionein-1 was located, but there was no accompanying PDB file. Several proteins were considered as surrogates for the structure for rabbit metallothionein-1, however, mouse metallothionein-1 was chosen as the

starting structure. All cysteine residues are conserved between mouse MT-1 and rabbit MT-1, and mouse MT-1 has 84% homology with rabbit MT-1. See Table 2.1 for comparison of rabbit and mouse metallothionein-1.

Accelrys' program Insight II was used for the molecular modeling experiments. The downloaded PDB files of mouse metallothionein-1 were separated into the alpha (C-terminus) and beta (N-terminus) domains. See Figures 2.1 and 2.2 for the model of the alpha and beta domains of mouse metallothionein-1.

Insight II was used to model the styrene oxide ligand. Figure 2.3 shows the structures for both styrene oxide and *trans*-phenylpropylene oxide. The builder module was utilized. An optimization was run on the structure to achieve a low-energy confirmation. First the docking procedure using the Affinity module was followed for docking styrene oxide to the mouse metallothionein-1 alpha domain followed by the beta domain. The styrene oxide was positioned close to the cysteine residues. The active site was defined for an area of a 6 Å radius. The docking was simulated in a vacuum with a dielectric constant of 1, and ten structures were accepted. Docking simulations were conducted using a dielectric constant of 80 to simulate water, and ten more structures were accepted. The initial minimization was 100 steps before the simulated annealing calculations. In the simulated annealing estimation the initial temperature was 500 °K cooling down to 300 °K. The final minimization was 1000 steps using the conjugate gradient method.

Alkylation and Digestion. To verify the modeling results and to experimentally determine where the styrene oxide adducted to the metallothionein, alkylation and digestion of the protein was necessary. Alkylation of the protein was required because of a tendency of the cysteine residues to form disulfide bonds, resulting in possible formation of false fragments after digestion. The alkylation and digestion procedure utilized was based on previous work (Hunziker, 1991) with modifications (Yu, 1995).

Samples for MT and MT-SO were prepared as previously described with the exception that a desalting column followed by lyophilization was utilized in place of the C18 solid phase extraction for metal removal.

For the metal removal using the desalting column, the column was first equilibrated with 20 mL of 0.1% (v/v) trifluoroacetic acid (TFA). The sample was then loaded onto the desalting column and eluted with 3.5 mL of 0.1% (v/v) TFA. Following elution, the sample was lyophilized.

The lyophilized protein was dissolved in 5 mL of buffer solution consisting of 6M guanidine HCl, 0.5 M tris/HCl, and 5 mM EDTA, pH 8.6, containing 10 mM DTT. The samples were then immersed in water bath at 37 °C for 2 hours. After incubation, 272 mM methyl 4-nitrobenzenesulfonate (2.5 mL) was added to the samples and they were again immersed in a water bath at 37 °C for an additional 6 hours. The reaction was stopped by acidification to pH 2.0 by addition of 20 % TFA, and the mixture was applied to a desalting column, and the eluant lyophilized.

The proteins were digested after dissolving the lyophilized samples in 0.1 M ammonium bicarbonate at 1 mg/mL. Trypsin was added at a 1:50 (w/w) trypsin/protein ratio. The samples were then incubated in a water bath at 37 °C for 2 hours. The digests were refrigerated, desalted as above, and lyophilized.

Mass Spectral Analysis. A Finnigan Deca LCQ quadrupole ion trap mass spectrometer was used for digest analysis. The source utilized electrospray ionization. Ions were monitored in the positive ionization mode. The samples were infused into the mass spectrometer at a flow rate of 7 μ L/min. The heated capillary temperature was 160 °C. The spray voltage was set to 5.21 kV, and the capillary voltage was at 50 V. The sheath gas flow rate was 39 mL/min.

Molecular Modeling. To further elucidate which cysteine residue was the site of adduct formation, molecular modeling experiments were conducted after the alkylation and digestion experiments. Using Insight II, styrene oxide adducts for both cysteine residues 44 and 48 were modeled using the downloaded mouse MT-1 file and assimilating the styrene oxide ligand to the cysteine residues in question. Next, a minimization using a combination of steepest descent and conjugate gradient for 1000 iterations was performed on each complex, and the resultant energies of the complexes were calculated.

3.1 Results

The major ions in the positive ion ESI spectrum of metallothionein were 1671 (+4 charge state), 1337 (+5 charge state), and 1115 (+6 charge state). This calculates to be a molecular weight of approximately 6685 for metallothionein (Fig. 3.1). The major charge distribution for MT was at the +6 charge state. After styrene oxide was adducted there was a shift in the spectrum such that the major ions became 1834 (+4 charge state), 1457 (+5 charge state), and 1230 (+6 charge state) (Fig. 3.2).

When metallothionein is taken through the metal removal process the mass spectrum simplifies. The major ions in the spectrum after metal removal were 1561 (+4 charge state), 1249 (+5 charge state), 1041 (+6 charge state), and 892 (+7 charge state). This calculates to be a molecular weight of approximately 6245 (Fig. 3.3). The major charge distribution for apo-MT was at the +5 charge state.

After adduct formation with styrene oxide, the MT sample went through the metal removal process to simplify the spectrum in an attempt to determine the stoichiometry of the styrene oxide to metallothionein reaction. At various charge states (+4 to +7), varying amounts of styrene oxide were adducted to the metallothionein (Fig. 3.4).

After adduct formation with *trans*-phenylpropylene oxide, the MT sample went through the metal removal process to simplify the spectrum in an attempt to see how much *trans*-phenylpropylene oxide was adducted to the metallothionein. At various charge states (+5 to +7), varying amounts of *trans*-phenylpropylene oxide were adducted to the metallothionein (Fig. 3.5). The mass comparisons of protein versus adducted protein are outlined in Table 3.1.

After adduct formation with styrene oxide, apo-MT was monitored at various charge states (+4 to +6), and styrene oxide was shown to be adducted to the apo-metallothionein (Fig. 3.6).

In an attempt to increase adduct formation between apo-MT and styrene oxide, dithiothreitol (DTT) was added to the reaction. Dithiothreitol is a reducing agent that minimizes the formation of disulfide bonds between cysteine residues and thereby

potentially increases adduct formation with styrene oxide by keeping the cysteine residues reduced and available for adduct formation. The results indicated better adduct formation after the addition of DTT (Table 3.1).

MS/MS experiments were conducted after adduct formation. Solutions of the non-adducted protein and adducted protein were infused separately into the mass spectrometer and subjected to varying amounts of collision energy. The change in mass from precursor to product ion were similar (m/z approximately 288) for the non-adducted protein and adducted protein for both SO and PPO (Table 3.2). When using increasing collision energies (30, 60, and 75 %) similar fragment patterns were also achieved. See Figures 3.7, 3.8, and 3.9 for MS/MS of metallothionein-I and the SO and PPO adducts at the +5 charge state.

For molecular modeling of metallothionein-I/styrene oxide adduct formation, the output from the runs was examined using the Insight II analysis module for alpha and beta domain adducts. The beta domain total energies were consistently lower than the alpha domain total energies (Table 3.3). According to the original hypothesis for the project the lower total energies should point to where adducts would most likely form. Next, protein-ligand bond distances were considered in adduct formation.

The protein-ligand bond distances considered were from the docking simulation where the dielectric constant was set to 80, simulating water. The experimental reaction was in water and so this particular simulation was chosen. Protein-ligand bond distances of less than 6 Å from a cysteine residue were considered possible sites for adduct formation. For several of the structures multiple cysteine residues met the criteria to be sites of adduct formation. Table 3.4 gives the residue site and protein-ligand bond distances for the Alpha domain. As can be seen from the table, A6, A7, and A18 (cysteine residues 36, 37, and 48 respectively) stand out for the most potential sites of adduct formation. In addition A14 (cysteine residue 44) also could be a site for adduct formation as the protein-ligand bond distances for 2 of the 3 matches were less than 4 Å.

Table 3.5 gives the protein-ligand bond distances for the beta domain. Only A5 (cysteine residue 5) is a potential site for adduct formation. Most calculated protein-

ligand bond distances for the beta domain were too long to support adduct formation with a cysteine site.

For the alkylation of the cysteine residues, various alkylating agents were tested. Full alkylation of metallothionein-I was achieved using N-ethylmaleimide. One very important factor in not incorporating N-ethylmaleimide into the final alkylation and digestion procedure was that the reaction is reversible (Shaw, 1997). The next alkylating agent tested was iodoacetamide (Bernhard, 1991). Iodoacetamide, however, only partially alkylated the protein. The final alkylating agent tested was methyl 4-nitrobenzenesulfonate. The alkylation with methyl 4-nitrobenzenesulfonate was still only partially complete, but to a greater extent than with the iodoacetamide.

The experimental confirmation of adduct formation suggests that formation occurs in the 44-51 peptide fragment of MT-1, which has an expected m/z of 825 (Table 3.6). When this fragment is successfully alkylated, the m/z of 867 corresponds to the three cysteine residues in the fragment being methylated (Fig. 3.10). Ions were observed at the expected m/z of 867 for the control sample (unadducted MT-I). An ion was observed at 973 for the SO adducted sample (Fig. 3.11). This ion corresponds to the addition of one SO and two methyl groups to the MT-I fragment 44-51 (Fig. 3.12). These experimental results were repeated and yielded the same result. This fragment result supports the modeling results for cysteine residue 48, or possibly cysteine residue 44.

The results from modeling the styrene oxide adducts on cysteine residues 44 and 48 of the alpha domain support the hypothesis of preferential adduct formation on residue 48 (see Fig. 3.13). After optimization, the van der Waals energy for adduct formation at cysteine residue 44 was 487.6 kcal/mol, as compared to 120.7 kcal/mol for cysteine residue 48. The total energy for adduct formation at cysteine residue 44 was 504.1 kcal/mol, as compared to 302.4 kcal/mol for cysteine residue 48.

3.2 Discussion

A characteristic of electrospray ionization is the spectrum represents an intact

molecule carrying differing numbers of charges. These ions arise from the molecule having multiple protonations or attachment of multiple cations (in positive ion mode). A shift in the charge state is possible, and can be the result of a change in solvent polarity or a loss of non-covalently bound atoms from the analyte resulting in a lower charge state distribution (Cole, 2000). A small charge state distribution shift was observed for MT versus apo-MT with a shift from the +6 to the +5 charge state, which could be due to loss of metals.

For styrene oxide, it was not determined in these experiments which carbon binds to the metallothionein. Either 1-phenyl-2-hydroxyethyl-MT or 2-phenyl-2-hydroxyethyl-MT could have formed.

Metallothionein forms adducts with styrene oxide (SO) and *trans*-phenylpropylene oxide (PPO). Adducts with SO and PPO were detected at all charge states identified for MT-I.

An additional experiment was conducted at this time under the previously described conditions to attempt to form adducts between styrene and metallothionein. MS experiments indicated no adduct formation between styrene and MT, as the spectrum remained unchanged from the control.

apo-Metallothionein forms an adduct with SO. For apo-metallothionein, the addition of reducing agent, DTT, showed increase of adduct formation with SO. In the presence of DTT the stoichiometry of styrene oxide to metallothionein increased to 3-4 molecules versus 1-2 molecules in the absence of DTT. apo-Metallothionein adducts with SO were detected at all charge states identified.

MS/MS experiments indicated the covalent nature of adduct formation of SO/PPO with metallothionein/apo-metallothionein. The change in mass from precursor to product ion is similar (m/z approximately 288) for the non-adducted and adducted protein for both SO and PPO. When using increasing collision energies (30, 60, and 75%) similar fragment patterns are achieved (Table 3.7), although the signal intensity lessens with increased collision energy. If the bond were non-covalent in nature, the loss of the styrene oxide mass would be expected at each charge state based on lower

dissociation energy associated with fragmenting non-covalent adducts in comparison with the energy required to fragment covalent bonds. The dissociation energy for a covalent C-N bond is 305 kJ/mol, and the dissociation energy for a covalent C-S bond is 280 kJ/mol. A loss corresponding to styrene oxide was not observed in any of the MS/MS experiments.

The identity of the MS/MS product ion loss (m/z approximately 288) is still not known. It is possible that the fragment is a -Met-Asp- fragment from the N-terminal end of metallothionein. Mammalian metallothioneins are characteristically N-acetylated (Beattie, 1999), and the presence of an acetyl group could yield a loss corresponding to an observed m/z loss of 288 with the formation of a ketene (Fig. 3.14).

The data from the molecular modeling lead to the conclusion that even though the energy is lower for the beta domain, adduct formation is more likely to occur in the alpha domain for mouse metallothionein-1. Four potential sites for adduct formation in the alpha domain were identified at cysteine residues 36, 37, 44 and 48. These simulations did not narrow the reactive site of the protein to a single cysteine residue.

A similar experiment was performed by Szilagyi and Fenselau using rat metallothionein-2 to form adducts with nitrogen mustard drugs (Szilagyi, 2000). The simulations also used Insight II. The cysteine residues at 33 and 48 in the alpha domain were found to be the most favorable for adduct formation. The criteria used were lower energy and bond lengths less than 4 Å.

Our calculations agree with the prediction of Szilagyi and Fenselau in that cysteine residue 48 is a favored site for adduct formation. In contrast cysteine residue 33 was not identified as a favored reaction site. Besides the obvious differences in proteins and ligands between the experiments, Szilagyi and Fenselau used explicit solvent information in their simulation. Their system was surrounded by water. This calculation method provides a better evaluation of electrostatic forces which are important in ligand-receptor interactions. Future experiments could be repeated using explicit solvent information to verify the prediction of the active sites for metallothionein.

The alkylation and protein digestion experiments indicate that adduct formation

occurs in the 44-51 peptide fragment of MT-1. Masses were observed at the expected m/z of 867 for the control sample (unadducted MT), and a mass was observed at 973 for the SO adducted sample indicating the addition of one SO. The experimental results are consistent with the modeling results. Further work to clarify adduct formation to the exact residue could be done using liquid chromatography with tandem mass spectrometry to separate peptide fragments and fully sequence them.

The results from modeling the styrene oxide adducts on cysteine residues 44 and 48 of the alpha domain again support the hypothesis of preferential adduct formation on residue 48. The residue with the lower total energy, cysteine residue 48, would be the more favorable site for adduct formation. In addition, from the modeling information, adduct formation on cysteine residue 48 is less sterically hindered than on cysteine residue 44 as the van der Waals energy differences indicate.

The stable nature of the adduct formation with SO and PPO with metallothionein and apo-metallothionein makes them ideal candidates as potential biomarkers.

4. Summary and Conclusions

Metallothioneins (MT) are intracellular, low molecular weight, cysteine-rich proteins that efficiently sequester epoxides, such as the genotoxic styrene metabolite, styrene oxide (SO). We hypothesized that covalent adduct formation by the ring-opening of SO and its homologue, *trans*-phenylpropylene oxide (PPO), with MT forms stable complexes that may serve as potential biomarkers for exposure to styrenes. MT was reacted with SO or PPO in water. After the MT reaction with SO and PPO, metals were removed prior to mass spectral analysis. Mass spectra were obtained under electrospray ionization conditions with positive-ion detection in an ion trap mass spectrometer. Mass spectra of MT-SO and MT-PPO showed that adducts were present in all charge states detected. MS/MS analysis indicated the covalent nature of the complexation. Further experiments were conducted to determine the site of adduct formation. Molecular modeling utilizing Insight II was used to predict the most likely site of adduct formation. Alkylation and digestion of MT and MT-SO were used to locate the peptide fragment

where adduct formation occurred and additionally confirm the modeling results. The likeliest cysteine residue for adduct formation was residue 48 in the alpha domain of MT-I. MT-styrene oxide adducts can provide a basis for the development of biomarkers for exposure to epoxides.

Table 2.1: Amino Acid Sequence Comparison of Mouse to Rabbit MT-1

Beta Domain			Alpha Domain		
Residue*	Type (mouse-1)	Type (rabbit-1)	Residue*	Type (mouse-1)	Type (rabbit-1)
A1 (1)	MET	MET	A1 (31)	LYS	LYS
A2 (2)	ASP	ASP	A2 (32)	SER	SER
A3 (3)	PRO	PRO	A3 (33)	CYS	CYS
A4 (4)	ASN	ASN	A4 (34)	CYS	CYS
A5 (5)	CYS	CYS	A5 (35)	SER	SER
A6 (6)	SER	SER	A6 (36)	CYS	CYS
A7 (7)	CYS	CYS	A7 (37)	CYS	CYS
A8 (8)	SER	ALA	A8 (38)	PRO	PRO
A9 (9)	THR	THR	A9 (39)	VAL	ALA
A10 (10)	GLY	GLY	A10 (40)	GLY	GLY
A11 (11)	GLY	ASN	A11 (41)	CYS	CYS
A12 (12)	SER	SER	A12 (42)	SER	THR
A13 (13)	CYS	CYS	A13 (43)	LYS	LYS
A14 (14)	THR	THR	A14 (44)	CYS	CYS
A15 (15)	CYS	CYS	A15 (45)	ALA	ALA
A16 (16)	THR	ALA	A16 (46)	GLN	GLN
A17 (17)	SER	SER	A17 (47)	GLY	GLY
A18 (18)	SER	SER	A18 (48)	CYS	CYS
A19 (19)	CYS	CYS	A19 (49)	VAL	ILE
A20 (20)	ALA	LYS	A20 (50)	CYS	CYS
A21 (21)	CYS	CYS	A21 (51)	LYS	LYS
A22 (22)	LYS	LYS	A22 (52)	GLY	GLY
A23 (23)	ASN	GLU	A23 (53)	ALA	ALA
A24 (24)	CYS	CYS	A24 (54)	ALA	SER
A25 (25)	LYS	LYS	A25 (55)	ASP	ASP
A26 (26)	CYS	CYS	A26 (56)	LYS	LYS
A27 (27)	THR	THR	A27 (57)	CYS	CYS
A28 (28)	SER	SER	A28 (58)	THR	SER
A29 (29)	CYS	CYS	A29 (59)	CYS	CYS
A30 (30)	LYS	LYS	A30 (60)	CYS	CYS
			A31 (61)	ALA	ALA

*The A designation is domain position, and the (number) designation is total protein position.
(Swiss-Prot, 2003)

Figure 2.1 Alpha Domain of Mouse Metallothionein-I (RSCB, 2003)

Figure 2.2 Beta Domain of Mouse Metallothionein-I (RCSB, 2003)

Figure 2.3 Styrene Oxide and *trans*-Phenylpropylene Oxide

Styrene Oxide [SO]

***trans*-Phenylpropylene Oxide [PPO]**

Table 3.1: Adduct Formation for MT-I with Styrene Oxide and *trans*-phenylpropylene oxide

Sample	Adduct Formation	+4 charge state (<i>m/z</i>)	+5 charge state (<i>m/z</i>)	+6 charge state (<i>m/z</i>)	+7 charge state (<i>m/z</i>)
MT	ND	1671	1337	1114.8	948.7
MT-SO	+2 SO	ND	1297	1081	ND
	+3 SO	1621	1321	1101	944
	+4 SO	1681	1345	1121	961
	+5 SO	1711	1369	1141	978
	+6 SO	1741	1393	1161	ND
	+7 SO	1771	1417	1181	ND
MT-PPO	+1 PPO	1594	1276	1063	911
	+2 PPO	1628	1303	1086	930
	+3 PPO	1661	1329	1108	950
	+4 PPO	1695	1356	1130	969
apoMT	n/a	1561	1249	1041	892
apoMT-SO	+1 SO	1591	1273	1061	ND
	+2 SO	1621	1297	1081	ND
apoMT-SO (DTT)	+1 SO	1591	1273	1061	ND
	+2 SO	1621	1297	1081	ND
	+3 SO	1651	1322	1101	ND
	+ 4 SO	1681	1345	n/a	ND

ND = not detected

Table 3.2: Product vs. Precursor Ions for Various Charge States of Metallothionein Adducts

Sample	+5 charge state		+6 charge state		+7 charge state	
	Precursor	Product	Precursor	Product	Precursor	Product
MT	1249	1191.5	1041	993.3	892	851.4
MT-SO	1297	1239.6	1081	1033.3	ND	ND
	1345	1287.5	1141	1093.3		
MT-PPO	1276	1218.5	1063	1016.1	911	870.6
	1303	1244.8	1086	1038.1		

ND = not detected

Table 3.3: Total Energies of Final structures for Alpha and Beta Adducts

Mouse-I Structure	Energy Alpha (vacuum) (kcal/mol)	Energy Beta (vacuum) (kcal/mol)	Energy Alpha (water) (kcal/mol)	Energy Beta (water) (kcal/mol)
1	437.4590	369.8630	344.4770	307.1810
2	440.9780	370.9230	346.6740	308.4490
3	441.3430	372.1730	349.5620	309.0830
4	442.6950	372.6940	350.0410	310.7720
5	444.3390	373.9290	352.2860	313.9340
6	444.4920	374.6710	353.2320	314.0260
7	444.7340	375.8580	355.6810	315.9610
8	447.8670	376.2370	355.9450	315.9990
9	450.4120	376.5850	359.0570	316.3760
10	455.1700	383.9900	359.4490	318.9410

Table 3.4: Optimal Protein-Ligand Distances in the α -Domain of less than 6 Å

Alpha mouse-1						
Structure	Residue/ length	Residue/ length	Residue/ length	Residue/ length	Residue/ length	Residue/ length
1	A4/2.55	A7/4.58	A14/3.06	A18/4.10		
2	A4/4.31	A6/5.87	A7/2.99	A14/4.52	A29/5.96	A30/5.42
3	A18/5.91					
4	A18/4.82					
5	A6/5.65					
6	A6/3.06	A7/5.87	A20/4.00	A29/4.28		
7	NONE					
8	A6/5.49	A14/3.18	A18/4.55	A30/4.38		
9	NONE					
10	A7/5.39	A27/4.72				

Table 3.5: Optimal Protein-Ligand Distances in the β -Domain of less than 6 Å

Structure	Residue/ length	Residue/ length	Residue/ length	Residue/ length
1	A5/4.22	A7/4.23	A24/5.86	A26/3.06
2	A5/4.75			
3	A5/4.43	A13/4.97	A26/4.40	
4	NONE			
5	NONE			
6	NONE			
7	A5/4.61			
8	NONE			
9	NONE			
10	NONE			

Table 3.6: Metallothionein-I Peptide Mapping

Peptide	Unalkylated m/z	Alkylated m/z
1-20 (MDPNCSCATGNSCTCASSCK)	1983	2053
21-22 (CK)	249	263
23-25 (ECK)	378	392
26-31 (CTSCKK)	669	697
32-43 (SCCSCCPAGCTK)	1162	1232
44-51 (CAQGCICK)	825	867 (+3 methyl) 973 (+2 methyl and +1SO)
52-56 (GASDK)	477	477
57-61 (CSCCA)	486	528

(Swiss-Prot, 2003)

Table 3.7: Collision Energy Comparison of Precursor vs. Product Ions at +5 Charge State

Sample	Precursor <i>m/z</i>	Collision Energy	Product Ions <i>m/z</i>	Signal Intensity
MT	1248.9	30%	1191.7	5.8×10^5
		60%	1191.9	1.7×10^5
		75%	1191.7	4.5×10^4
MT-SO	1297.6	30%	1239.3	1.0×10^5
		60%	1239.1	4.1×10^4
		75%	1240.0	2.7×10^4

Figure 3.1 Mass Spectrum of Metallothionein-I

Figure 3.2 Mass Spectrum of Metallothionein-I/Styrene oxide Adduct

Figure 3.3 Mass Spectrum of apo-Metallothionein-I

Figure 3.4 Mass Spectrum of MT-I/SO Adduct after metal Removal

Figure 3.5 Mass Spectrum of MT-I/PPO Adduct after Metal Removal

Figure 3.6 Mass Spectrum of apoMT-I/SO Adduct

Figure 3.7 MS/MS of Metallothionein-I at +5 Charge State
(Precursor 1249)

Figure 3.8 MS/MS of MT-I/SO Adduct at +5 Charge State
(Precursor 1273)

Figure 3.9 MS/MS of MT-I/PPO Adduct at +5 Charge State
(Precursor 1276)

Figure 3.10 Mass Spectrum of Metallothionein-I Digest

Figure 3.11 Mass Spectrum of MT-I/SO Adduct Digest

Figure 3.12 Proposed Structure for SO Adducted Peptide Fragment 44-51 for MT

Figure 3.13 Proposed Metallothionein-I/Styrene oxide Adduct at Cysteine Residue 48

Figure 3.14 Proposed Structure of MS/MS Fragment Corresponding to Loss of 288

5. Reference List

Antoine M, Fabris D, and Fenselau C: Covalent sequestration of the nitrogen mustard mechlorethamine by metallothionein. *Drug Metabolism and Disposition* 1998; (26), 921-926.

Beattle J, Wood A, Duncan G: Rat metallothionein-2 contains N-acetylated and unacetylated isoforms. *Electrophoresis* 1999; (20), 1613-1618.

Bernhard W: Differential modification of metallothionein with iodoacetamide. *Methods in Enzymology* 1991; (205), 426-433.

Dabrio M, Rodriquez A, Bordin G, Bebianno M, DeLey M, Sestakova I, Vasak M, Nordberg M: Recent developments in quantification methods for metallothionein. *Journal of Inorganic Biochemistry* 2002; (88), 123-134.

Cole R: Some tenets pertaining to electrospray ionization mass spectrometry. *Journal of Mass Spectrometry* 2000; (35), 763-772.

Gehrig P, You C, Dallinger R, Gruber C, Brouwer M, Kagi J, and Hunziker P: Electrospray ionization mass spectrometry of zinc, cadmium, and copper metallothioneins: evidence for metal-binding cooperativity. *Protein Science* 2000; (9), 395-402.

Hathout Y, Reynolds K, Szilagyi Z, Fenselau C: Metallothionein dimmers studied by nano-spray mass spectrometry. *Journal of Inorganic Biochemistry* 2002; (88), 119-122.

He T, Wei D, Fabris D, and Fenselau C: Intracellular sequestration of anti-tumor drugs by metallothionein. *Cellular and Molecular Biology* 2000; (46), 383-392.

Hong S, Toyama M, Maret W, and Murooka Y: High yield expression and single step purification of human thionein/metallothionein. *Protein Expression and Purification* 2001; (21), 243-250.

Hunziker P: Cysteine modification of metallothionein. *Methods in Enzymology* 1991; (205), 399-400.

Ishida T, Kumagai Y, Ikeda Y, Ito K, Yano M, Toki S, Mihashi K, Fujioka T, Iwase Y, and Hachiyama S: (8S)-(Glutathion-S-YL)dihydromorphinone, a novel metabolite of morphine from guinea pig bile. *Drug Metabolism and Disposition* 1989; (17), 77-81.

Koh M, and Kim H: The effects of metallothionein on the activity of enzymes involved in removal of reactive oxygen species. *Bulletin of the Korean Chemical Society* 2001; (22), 362-366.

Linhart I: Stereochemistry of styrene biotransformation. *Drug Metabolism Reviews* 2001; (33), 353-367.

Nordberg G, Nordberg M, Piscator M, Vesterberg O: Separation of two forms of rabbit metallothionein by isoelectric focusing. *Biochemical Journal* 1972; (126), 491-498.

Prange A, Schaumlöffel D: Hyphenated techniques for the characterization and quantification of metallothionein isoforms. *Analytical and Bioanalytical Chemistry* 2002; (373), 441-453.

Price E, Smith J, Clark C, Schlager J and Shih M: MALDI-TOF/MS as a diagnostic tool for confirmation of sulfur mustard exposure. *Journal of Applied Toxicology* 2000; (20), S193-S197.

Research collaboratory for structural bioinformatics (RCSB), protein data bank: Rutgers, State University of New Jersey. <http://www.rscb.org/pdb/>. 2003.

Rigby K, Stillman M: Structural studies of metal-free metallothionein. *Biochemical and Biophysical Research Communications* 2004; (325), 1271-1278.

Sanz-Nebot V, Andon B, Barbosa J: Characterization of metallothionein isoforms from rabbit liver by liquid chromatography coupled to electrospray mass spectrometry. *Journal of Chromatography B* 2003; (796), 379-393.

Sato M, Hida M, Nagase H. Analysis of pyrolysis products of dimethylamphetamine. *Journal of Analytical Toxicology* 2001; (25), 304–309.

Shaw C, He L, Munoz A, Savas M, Chi S, Fink C, Gan T, Petering D: Kinetics of reversible N-ethylmaleimide alkylation of metallothionein and the subsequent metal release. *Journal of Biological Inorganic Chemistry* 1997; (2), 65-73.

Swiss-prot protein knowledgebase: ExPASy (expert protein analysis system) proteomics server of the Swiss institute of bioinformatics (SIB). <http://au.expasy.org/sprot/sprot.top.html>. 2003.

Szilagyi Z, Fenselau C: Molecular dynamics simulation of metallothionein-drug complexes. *Drug metabolism and Disposition* 2000; (28), 174-179.

Watabe T, Hiratsuka A, Ozawa N, Isobe M: Glutathione S-conjugates of phenyloxirane. *Biochemical Pharmacology* 1981; (30), 390-392.

Wei D, Fabris D, and Fenselau C: Covalent sequestration of phosphoramidate mustard by metallothionein- an in vitro study. *Drug Metabolism and Disposition* 1999; (27), 786-791.

Yu X, Zhuchun W, and Fenselau C: Covalent sequestration of melphalan by metallothionein and selective alkylation of cysteines. *Biochemistry* 1995; (34), 3377-3385.

Zaia J, Fabris D, Wei D, Karpel R, and Fenselau C: Monitoring metal ion flux in reactions of metallothionein and drug-modified metallothionein by electrospray mass spectrometry. *Protein Science* 1998; (7), 2398-2404.

Zaia J, Jiang L, Han M, Tabb J, Wu Z, Fabris D, and Fenselau C: A binding site for chlorambucil on metallothionein. *Biochemistry* 1996; (35), 2830 – 2835.

Zangger K, Shen G, Oz G, Otvos J, Armitage I: Oxidative dimerization in metallothionein is a result of intermolecular disulphide bonds between cysteines in the α -domain. *Biochemical Journal* 2001; (359), 353-360.