COVALENT SEQUESTRATION OF PHOSPHORAMIDE MUSTARD BY METALLOTHIONEIN—AN IN VITRO STUDY

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ABSTRACT:

Acquired drug resistance is one of the most important problems in cancer chemotherapy. One of the proposed mechanisms for these phenomena is the sequestration of alkylating agents by metallothionein in vivo. This research shows that metallothionein can covalently sequester phosphoramide mustard, the active form of cyclophosphamide in vitro. On-line electrospray mass spectrometry reveals that it is phosphoramide, not nornitrogen mustard that alkytes metallothionein, although the metallothionein/nornitrogen mustard adduct was isolated as the major adduct. Tandem mass spectrometric experiments were performed on an isolated drug-modified tryptic peptide. The alkylation occurred predominantly at Cys48 of metallothionein. These results provide further evidence that overexpression of metallothionein can detoxify the active form of the drugs.

Metallothionein (MT)\(^1\) is a family of small metal-binding proteins that exists ubiquitously in all kinds of cells in mammalians. MT was first identified in equine kidney cortex in 1957 (Margoshes and Vallee, 1957). It has a low molecular weight (usually 61–62 amino acids in a single polypeptide chain) and high cysteine content (20 cysteines, ~30%). In addition, each MT molecule binds seven Zn\(^{2+}\) and Cd\(^{2+}\) ions (Otvos et al., 1985). MT occurs in diverse isoforms. Humans have the most complicated MT isoform family, which includes human MT-1a, 1b, 1e, 1f, 1g, 1 h, 1x; human MT-2; human MT-3; and human MT-4 (Moffatt and Denizeau, 1997). These MT isoforms usually share highly conserved sequences with almost invariant positions of their 20 cysteines. One of the most remarkable features of MT is its inducibility both in vivo and in cultured cells. The MT concentration in tissues is usually highly variable and changes significantly under the influence of a large variety of conditions, including metal ions such as Zn\(^{2+}\), Cd\(^{2+}\), Cu\(^{2+}\), Hg\(^{2+}\), antibiotics, hormones, inflammatory agents, anticancer drugs, and stress-producing conditions (Kagi, 1993; Moffat and Denizeau, 1997).

MT has two discrete domains. Rat liver Cd\(_2\)MT 2a can be cleaved by protease into two domains (Winge and Miklosy, 1982): a \(\beta\) domain at the N terminal that contains a Cd\(_4\)Cysh metal cluster and an \(\alpha\) domain at the C terminal that contains a Cd\(_4\)Cys\(_{10}\) cluster. X-ray crystallography shows that each domain has a solvent accessible cleft (Robbins et al., 1991). The cleft in the \(\beta\) domain contains Cys5, Cys7, and Cys13 as solvent-exposed residues, whereas the cleft in the \(\alpha\) domain contains Cys37, Cys41, and Cys57 as solvent-exposed residues.

Although MT has been extensively studied in the past 40 years, its exact function remains a topic of discussion (Bremner, 1991). One proposed function of MT is detoxification of heavy metals such as cadmium and mercury (Kagi and Vallee, 1961). It is suggested that MT plays a key role in the storage and regulation of Zn\(^{2+}\) and Cu\(^{2+}\) in vivo (Bremner, 1991). It may also regulate transcription by means of Zn\(^{2+}\) transfer to nucleic acid-binding proteins (Zeng et al., 1991). The Zn\(^{2+}\) transfer of MT can be modulated by the glutathione redox couple (Jiang et al., 1998). Although transgenic mice carrying no functional MT genes grew and reproduced normally (Michalska, 1993), they did show increased sensitivity to chemical stress (Lazo et al., 1995).

Recently, much work has been focused on the relation between MT induction and acquired drug resistance during cancer chemotherapy. Accumulated evidence supports the idea that overexpression of MT contributes to acquired drug resistance in chemotherapy by alkylating agents (Lazo and Basu, 1991). Human tumor cell lines with induced high levels of MT have been found to gain resistance to anticancer drugs (Kelly et al., 1988). Human tumor cell lines with elevated MT induced by heavy metals showed increased resistance to cis-dichlorodiaminediphosphate and nitrogen mustards (Basu and Lazo, 1990). Overexpression of MT has also been found in tumor cells from cancer patients after chemotherapy (Wood et al., 1993). It was found that the alkylating agents melphalan (Yu et al., 1995), chlorambucil (Zaia et al., 1996), and mechlorethamine (Antoine et al., 1998) reacted covalently with MT under near physiological conditions in vitro. The major alkylation sites were Cys48 and Cys33. Molecular modeling supports selective binding of these drugs. When human bladder tumor T24 cells were treated with chlorambucil, multiple MT alkylation occurred (T. He, D.F. and C.F., submitted). In addition, it was also found that chlorambucil-alkylated MT transfers zinc ions to other protein more rapidly than unmodified MT, which may trigger the overexpression of MT by means of gene regulation (Zaia et al., 1998).
In this study, the reaction of phosphoramide mustard (PM), the active form of cyclophosphamide (CP), with MT was investigated to provide a further test of the hypothesis.

**Experimental Procedures**

**Materials.** Rabbit liver MT 2a (Zn 0.7%, Cd 5.3%, Lot#34H95161) was purchased from Sigma Chemical Co. (St. Louis, MO). Bovine trypsin (TPCK-treated), zinc atomic absorption standard solution, methyl-4-nitrobenzenesulfonate, tris(hydroxymethyl)aminomethane, dithioerythritol, trifluoroacetic acid (TFA), and guanidine hydrochloride were also supplied by Sigma. HPLC grade acetonitrile was obtained from J. T. Baker Chemical Company (Phillipsburg, NJ). A five thousand molecular weight cut off centrifugal filter device was purchased from Millipore (Fisher Scientific, Pittsburgh, PA). PM was obtained from the Drug Synthesis & Chemistry Branch, National Cancer Institute (Bethesda, MD).

**Mass Spectrometry (MS) Analysis.** Matrix-assisted laser desorption ionization (MALDI) MS was performed using a Kratos Kompact MALDI III mass spectrometer (Manchester, UK) with a 337-nm nitrogen laser. The matrix mixture used was 3,5-dimethoxy-4-hydroxycinnamic acid and α-cyano-4-hydroxycinnamic acid. (Aldrich, Milwaukee, WI). Both linear and reflectron modes were used with external calibration.

Electrospray mass ionization (ESI) experiments were performed on the first mass spectrometer of a JEOL Ltd. (Tokyo, Japan) HX110/HX110 four-sector mass spectrometer equipped with a thermally assisted electrospray source from Analytica of Branford (Branford, CT). Resolution was set at 500 (10% valley) by adjusting the slit widths and the mass accuracy was 0.03% or better. Mass spectra were infused into the source at 1 μl/min using a Harvard syringe pump (Harvard Apparatus, South Natick, MA) and the interfacing capillary was heated to 120°C. For the ESI experiments under acidic conditions, a solution of H2O/methanol (MeOH)/AcOH (49:49:2 by volume) was used. The analyte was either dissolved directly in the solution or dissolved first in H2O and then mixed with MeOH and acetic acid. For the ESI experiments under neutral conditions, 10 mM NH4 HCO3 was used and a sheath flow of MeOH was sprayed coaxially to the analyte solution at 1 μl/min. The spray stainless steel needle was kept at ground potential and the counter-electrode at 4kV.

Fast atom bombardment (FAB) experiments were carried out on the first mass spectrometer of the JEOL HX110/HX110 mass spectrometer. The FAB gun was operated at 6 kV and the accelerating voltage was 10 kV. The resolution was set at 1000 (10% valley) by adjusting the slit widths. Spectra were calibrated by the data system against a FAB spectrum of cesium iodide clusters and the mass accuracy was 0.03% or better.

**Reconstitution of Zn7 MT.** Zn0 MT was prepared according to an earlier reference (Vasak, 1991). Briefly, the commercial Zn(Cd) MT was dissolved in 50 mM HCl, desalted by gel filtration, and further purified by a size-exclusive column. The apo-MT was collected, lyophilized, and stored at −20°C. For reconstitution, purified apo-MT was dissolved in degassed 50 mM HCl (0.5 mg/ml) containing dithioerythritol (0.25 mg/ml) under argon atmosphere. Eight equivalents of ZnCl2 solution (15.4 mM in 1% HCl) was added and the solution was brought to pH 7.5 by slowly adding 0.5 M tris(hydroxymethyl)aminomethane. The reconstituted Zn7 MT2a was desalted and changed to 10 mM NH4 HCO3 buffer using Millipore (5000 MW cut) ultrafiltration. The Zn7 MT concentration was determined by measuring the UV absorbance at 220 nm of the protein in 100 mM HCl solution (ε = 48,200 mol−1 cm−1; Vasak, 1991).

**In Vitro Alkylation of MT.** Cd/Zn MT2a (586 μM, 1 equivalent) and PM (2.05 mM, 3.5 equivalents) were incubated in 100 mM potassium phosphate buffer (pH 7.4) at 37°C for 15 min. The reaction mixtures were acidified to pH 2 by adding 20% TFA and desalted by gel filtration. The collected fraction was frozen and lyophilized before mass spectrometric analysis or HPLC separation.

**Methylation of the Alkylated MT.** The alkylated MT was stabilized by cysteine methylation (Hunziker, 1991). The methylation reaction was stopped by adding 20% TFA to pH 2 and desalted by gel filtration.
15 min, the majority of MT was still unreacted (72% of apo-MT2a and apo-MT2a' by integration). About 28% of MT was alkylated. Products included one major alkylation product (46.5 min, 23% by integration) and some minor products (49.0 min, 5% total by integration). The alkylation of MT increased with higher reactant concentrations, longer reaction time, or more equivalents of PM (data not shown); however, the alkylated products tended to deteriorate under vigorous conditions and no distinct products were detected by HPLC or ESI MS.

Identification of Alkylated Products. The isolated products were analyzed by ESI MS under acidic conditions (Fig. 2). Table 1 summarizes the identified alkylated products and their structures, determined by their average molecular masses. The molecular mass of the rabbit liver MT2a isoform was measured as 6122.8 Da, which agrees well with the reported mass (M_{MT2a} = 6125.3 Da; Yu et al., 1993). The other MT isoform MT2a' was determined as 6155.6 Da (M_{MT2a'} = 6155.3; Yu et al., 1995). The major product had a molecular mass of 6230.0 Da, which was determined as the apo-MT2a isoform covalently bonded to one nornitrogen mustard (NNM; apo-MT2a-NNM, calculated MW: 6230.8 Da). It is interesting that the phosphoramidate group was lost in this adduct. Experiments were carried out to determine whether hydrolysis happened before the alkylation or during the reaction work up (see below). The minor products were determined to be apo-MT2a mono PM adduct (apo-MT-PM) and the same adduct with the chloride group hydrolyzed to a hydroxyl group (apo-MT-PM(-OH)). The relative amounts of the minor adducts varied depending on the conditions used for stopping the reaction and separation. The work up condition was controlled to get apo-MT-NNM as the major product.

Mapping the Alkylation Site by FAB and ESI MS. Protein mapping by tryptic digestion and peptide sequencing by MS/MS experiments were carried out to determine which cysteine(s) were alkylated by the drug. The alkylated products were methylated and digested by trypsin without prior HPLC separation (see Experimental
Procedures. The tryptic peptides were separated by RP-HPLC and fractions were checked by MALDI, FAB, and ESI MS experiments and compared with control experiments. The results of FAB MS experiments are summarized in Table 2. A major modified peptide was found to be alkylated peptide [44-51] (CAQGCICK), with a mass increase of 99 Da. No other modified peptides were detected by HPLC or MS.

Sequencing of Alkylated Peptide. The alkylated peptide [44-51] was purified and collected by HPLC. MS/MS experiments were carried out to sequence the peptide (Fig. 3). The immonium ions (I, C, Q, C" drug) and fragment 805.2 Da, which also appeared in the control MS/MS experiment of unmodified peptide [44-51] (data not shown), confirmed the peptide as [44-51] with a drug molecule covalently bound to one of its cysteine side chains. The w ion series was used to determine unequivocally that the drug was linked to Cys48 of apo-MT2a isoform. The mass of the drug group was determined to be 114 Da. The structure of this group was determined as ethyl-2-oxazolinone (Fig. 5). This structure has also been found in PM metabolic studies (Momerency et al., 1994; Joqueviel et al., 1998).

MT-PM (OH)

MW, average molecular weight.

TABLE 2
FAB MS of tryptic peptides of methylated rabbit liver MT2a-NNM adduct

<table>
<thead>
<tr>
<th>Peptides</th>
<th>m/z Calculated</th>
<th>m/z Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>01-22</td>
<td>2382.1</td>
<td>2383.6</td>
</tr>
<tr>
<td>23-25</td>
<td>334.4</td>
<td>335.6</td>
</tr>
<tr>
<td>26-30</td>
<td>697.3</td>
<td>697.6</td>
</tr>
<tr>
<td>32-43</td>
<td>1228.4</td>
<td>1228.8</td>
</tr>
<tr>
<td>44-51 (+drug)</td>
<td>867.3</td>
<td>966.7</td>
</tr>
<tr>
<td>52-56</td>
<td>477.2</td>
<td>477.6</td>
</tr>
<tr>
<td>57-61</td>
<td>528.4</td>
<td>528.5</td>
</tr>
</tbody>
</table>

The sequence of rabbit liver MT-2a, AcMDPNCSCAAAG10-DSCCTCANSCT15CCKAC-KCTSCK16KSCCSCCPC97C4AK-AQGCIC9KGASDKSCC96A (Vasak et al., 1987).

reaction, the reaction was carried out in a methylation buffer without 6 M guanidine under argon atmosphere. For the tryptic digestion, a buffer of potassium phosphate of the same pH was used instead of ammonium bicarbonate and the reaction was also carried out under argon atmosphere. Under these conditions the alkylated peptide was found to give a mass of 926 by MALDI, corresponding to peptide [44-51] bound to a molecule of NNM that has eliminated HCl (Zaia et al., 1996).
Electrospray MS was used to determine the nature of the alkylating reagent. The reaction between MT and PM was carried out at pH 7 in a solution infused directly into the electrospray ionization source.

The results are shown as Fig. 4. The major product under these conditions was found to be Zn₇MT-PM (Fig. 4; observed: 6758.0Da, calculated: 6753.2). No Zn₇MT-NNM was detected.

Discussion

CP is the most widely used alkylating agent in tumor chemotherapy (Chabner and Collins, 1990). Unlike many other nitrogen mustards, CP shows almost no cytotoxic activity in vitro. It is a prodrug that needs to be metabolically activated (Hill, 1975). In vivo, nontoxic CP is oxidized to a cytotoxic species, 4-hydroxycyclophosphamide, by an enzyme system including cytochrome P-450-linked oxidase. The oxidation takes place predominantly in microsomes in the liver (Brock, 1971). The activated 4-hydroxycyclophosphamide undergoes isomerization and elimination to form PM and acrolein.

Many CP metabolites have been found (Colvin et al., 1973; Joqueviel et al., 1998). Among them, 4-hydroxycyclophosphamide and PM have gained much attention. Although both show high cytotoxic activities, they are suggested to play different roles in vivo (Alberts et al., 1984). The 4-hydroxycyclophosphamide species is regarded as a transportation form, entering cells in conjugation with other molecules. PM is considered to be the active form of CP, alkylating biopolymers intracellularly. These characteristics suggest that although CP is used in in vivo pharmacological studies, 4-hydroxycyclophosphamide is a good candidate in a study using a cell line and PM is a good candidate for test tube studies. Although the interaction of these two species with glutathione has been extensively studied (Pallante et al., 1986; Dirven et al., 1994), references on their interaction with proteins are rare. It has been reported that although CP was minimally protein-bound (13%), its metabolites were bound by plasma protein as much as 56% (Jardine et al., 1978).

In this study, PM was found to react readily with MT under near physiological conditions in vitro. Under the reaction and work up conditions used, the major product is apo-MT-NNM (Table 1). Direct ESI-MS experiments proved that the hydrolysis of PM to NNM happened during the acidic work up conditions. Unlike melphalan and chlorambucil, PM does not have an aromatic chromophore. Thus, the hydrophobicities of the drug-modified MTs do not change much on the HPLC chromatogram (Fig. 1), similar to the adducts of MT with mechlorethamine (Antoine et al., 1998).

Previous studies (Yu et al., 1995; Zaia et al., 1996; Antoine et al., 1997) showed that alkylating agents react with rabbit liver MT₂a selectively at Cys₄₈ and Cys₃₃. The selectivity is proposed to result presumably from a noncovalent binding of the aziridinium cation of the drug to a pocket near Cys₄₈ of MT. Molecular dynamics and solid-docking simulations confirmed that the binding pocket is within a region bordered by the two domains and the linking region of MT. In agreement with these studies, the exclusive binding site of MT by PM has also been proven to be Cys₄₈. The biological significance of these interesting results is still unknown. Alkylation of the Cys₃₃ site in the MT was not detected in this study. Compared with the chemical structures of other alkylating agents such as mechlorethamine, the unique phosphoramidate anion group in PM may interact with the electron-rich region near the binding pocket, which will affect both its reaction rate and selectivity. When the results of HPLC quantitation and on-line ESI/MS experiments are compared, the relative reaction rates of MT alkylation by nitrogen mustards are: PM < melphalan < chlorambucil < mechlorethamine. Although not a rigorous kinetic study, this order agrees with the reaction kinetics with guanosine (Kallama and Hemminki, 1984) and 4-(p-nitrobenzyl)pyridine (Bardos et al., 1965).

When PM reacted with ethanethiol at near physiological conditions, both a PM adduct and a NNM adduct were identified (Colvin et al., 1976). It was argued that PM, not NNM, acted as the alkylation agent,
because NNM does not show alkylating activity at pH 7.4 with 4-(p-nitrobenzyl)pyridine assay. Our on-line ESI/MS detection of PM/MT reaction provides direct evidence that PM forms a covalent bond with MT first and that the PM adduct may be hydrolyzed to a NNM adduct, especially under acidic conditions.

As shown in Fig. 5, the alkylation adduct PM/MT hydrolyzes to NNM/MT rapidly upon acidification. The NNM/MT adduct reacts readily with trace amounts of carbon dioxide to form the ethyl-2-oxazolone adduct at slightly basic pH. These two derivatives of PM, although obtained during work-up, agree well with previous in vivo and in vitro studies of PM metabolism and decomposition (Williamson et al., 1966; Struck et al., 1975; Colvin et al., 1976; Momerency et al., 1994; Joquevel et al., 1998). However, under argon atmosphere a β elimination (—HCl) derivative was formed (Zaia et al., 1996).

This study provides the evidence that during cancer chemotherapy by CP, the intracellular MT may sequester the active form PM and reduce its concentration. The results provide further evidence to support the hypothesis that overexpressed MT will confer tumor cell resistance to nitrogen mustards administered therapeutically.

References

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