Covalent Sequestration of Melphalan by Metallothionein and Selective Alkylation of Cysteines†

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ABSTRACT: Rabbit liver metallothionein-2 is shown to form covalent bonds with the anticancer agent melphalan, in support of the hypothesis that covalent sequestration by metallothionein constitutes one mechanism for the cross-resistance acquired by cancer patients to therapeutic alkylating agents. Among 20 cysteines in the 2-domain protein, 89% of the first alkylation reaction occurs with 2 that cochelate a zinc cation in the carboxy domain. Computer-supported docking studies indicate a favorable binding site for melphalan near these cysteine sulfhydryl groups. Although folded metallothionein-2 is resistant to trypsin cleavage, alkylation by 1 mol of melphalan allows cleavage by trypsin between the two globular domains.

In the defense against exogenous chemicals, cells can activate a wide spectrum of biochemical mechanisms that ultimately result in reduced sensitivity or resistance toward these compounds (Hayes & Wolf, 1990). Sequestration of xenobiotics, among other processes by which cells reduce the amount of harmful chemicals, is one effective mechanism to protect critical cellular functioning from chemical invasion. The increased expression of metallothionein (MT)[1] has been implicated in this mechanism. Studies in both cultured cells and animals have demonstrated that cells can at least temporarily attenuate the cytotoxicity of cadmium and other heavy metals by MT gene amplification (Beach & Palmiter, 1981). Elevated levels of MT correlate with the reduction of the toxicity of various metal ions and some alkylating agents (Bakka et al., 1981; Endresen et al., 1983). Radioactivity measurements suggested that intracellular MT sequesters these alkylating agents and thus contributes to the resistance against these drugs. Cells selected with resistance against cadmium displayed cross-resistance to several alkylating agents, such as *cis*-diamminedichloroplatinum, chlorambucil, melphalan, merchlorethamine, and 4-hydroperoxycyclophosphoramide (Teicher et al., 1987; Kelley et al., 1988). It has been shown unequivocally that MT is involved in the resistance against alkylating agents, as mouse cells transfected with the human MT-IIA gene were found resistant to melphalan and chlorambucil. No increase in nonprotein thiols was found in these studies, supporting the hypothesis that intracellular MT sequesters these alkylating agents.

However, it is not clear at a molecular level how cells or organisms benefit from increased intracellular MT in the defense against exogenous chemicals (Lazo & Basu, 1991; Kaina et al., 1990). Direct sequestration in cytosol would result in reduced amounts of alkylating agents available for their intended DNA target, especially when the protein level can be increased 10−20-fold due to induction. Alternatively, MT may undergo physical and structural changes upon reaction with alkylating agents and other xenobiotics. This may further affect the intracellular distribution of metal ions and trigger multiple biochemical responses. It has been suggested that MT may serve as an intracellular zinc regulatory factor and control metal homeostasis in cell replication, transcription, and translation (Karin, 1985; Vallee, 1991). Although MT is thermodynamically stable (Vašák & Kägi, 1983), it is kinetically labile as metal ions in solution and in MT clusters exchange rapidly (Nettesheim et al., 1985). ApoMT was shown to modulate DNA binding and transcription activation in vitro by competing for zinc ions with zinc finger transcription factors (Zeng et al., 1991a,b). Treatment of MT with oxidized glutathione resulted in intramolecular disulfide formation and the release of metals originally bound in MT clusters (Wolfgang, 1994). A putative glutathione binding site in rabbit liver MT was suggested recently, based on equilibrium binding and molecular modeling studies (Brouwer et al., 1993).

The exact biological function of MT is still under discussion. The biosynthesis of the protein is induced transcriptionally by a wide range of chemical agents and physical conditions, including heavy metals, alkylating agents, hormones, growth factors, tumor promoters and oncogenes, interleukins and interferons, and radiation (Hamer, 1986; Palmiter, 1987; Kägi, 1991). As a family of low molecular weight metal−thiolate proteins, MT occurs ubiquitously in animals (Kägi & Schaffer, 1988). Mammalian MTs contain 61 or 62 amino acid residues, with 20 cysteines deprotonated and coordinated to a total of 7 divalent metal ions (mostly Zn and Cd). Two separate metal−thiolate clusters are formed when the protein is fully folded, 1 with 4 metal and 11 Cys (α-domain) in the C-terminal half, and the other with 3 metal and 9 Cys (β-domain) in the N-terminal half (Otvos & Armitage, 1980; Winge & Miklossy, 1982). Nuclear magnetic resonance (NMR) studies revealed that cluster formation and polypeptide folding in MT are determined by the conserved arrangement of the 20 Cys (Frey et al., 1985; Arseniev et al., 1988). For MT-2

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[1] Abbreviations: MT, Metallothionein; TFA, trifluoroacetic acid; ESI-MS, electrospray ionization mass spectrometry; FABMS, fast atom bombardment mass spectrometry; MS/MS, tandem mass spectrometry; GSH, glutathione.

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from human, rabbit, and rat liver, all 28 metal−polypeptide coordinative bonds were found to be identical, and the overall conformations of these proteins are very similar (Messerle et al., 1990).

In this study, we demonstrate directly that rabbit liver MT-2 sequesters melphalan and forms 1:1 covalent adducts at physiological pH (Yu et al., 1993) and intracellular melphalan concentration (Bolton et al., 1993). Six cysteines have been identified as melphalan alkylation sites, with two of them accounting for almost 90% of the alkylation. Metallothionein alkylated by 1 mol of melphalan displays an increased but limited trypsin susceptibility, indicating a conformational change.

EXPERIMENTAL PROCEDURES

Rabbit liver metallothionein (MT-2) and melphalan were purchased from Sigma Chemical Co. (St. Louis, MO). Trifluoroacetic acid (TFA), tris(hydroxymethyl)aminomethane (Tris), methyl-4-nitrobenzenesulfonate, and guanidine hydrochloride were also purchased from Sigma. Trypsin (TPCK-treated) was from Sigma, and endoproteinase Asp-N was purchased from Boehringer Mannheim (Mannheim, Germany). Acetonitrile (HPLC grade) was purchased from J. T. Baker (Philipsburg, NJ), and water was deionized to 18 MΩ by an Ionpure system (Landover, MD).

Commercial MT and reconstituted Cd_7-MT samples were used in alkylation studies. The procedure for preparation of reconstituted Cd_7-MT was described previously (Vašák, 1991; Yu et al., 1993). MT (10 mg) was dissolved in 10 mL of potassium phosphate buffer (0.1 M, pH 7.4), and the solution was then added to a glass vial containing melphalan (420 μg). The reaction mixture was incubated at 37 °C with stirring for 30 min. The molar ratio of MT to melphalan was 1:1 with a concentration of 140 μM. The reaction mixture was acidified to pH 2 by addition of 20% (v/v) TFA/H_2O, and the metals were released upon complete denaturation of the protein. Metal-free MT was isolated from the reaction mixture by gel filtration using a Sephadex G-25M column (Pharmacia PD-10, Uppsala, Sweden) equilibrated with 0.1% TFA/H_2O. After lyophilization, the dry protein was analyzed by reversed phase high-performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS).

The lyophilized mixture of metal-free MT and melphalan-alkylated MT (MT-mel) was methylated at all free thiol positions using a modified procedure described previously (Hunziker, 1991b). Approximately 9 mg of the protein was dissolved in 10 mL of reaction buffer [6 M guanidine hydrochloride, 0.5 M Tris/HCl, and 5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.6] with 10 mM dithiothreitol (1.5 mg/mL). Methyl-4-nitrobenzenesulfonate was dissolved in acetonitrile at 272 mM (59 mg/mL), and 5 mL was added to the MT solution with mixing. The reaction mixture was incubated under argon for 1 h at 37 °C, and the methylation was stopped by addition of 20% TFA/H_2O to bring the pH below 2. The S-methylated mixture of MT and MT-mel (MMT and MMT-mel, respectively) was recovered by gel filtration with a Sephadex G-25 column (Pharmacia PD-10) equilibrated with 0.1% TFA/H_2O. The completion of methylation was checked by ESI-MS after samples were lyophilized to dryness.

Methylated MT and MT-mel were dissolved in 0.1 M ammonium bicarbonate buffer at pH 8 with a final concentration of 0.5 mg/mL. Trypsin (TPCK-treated) was dissolved in 0.1 mM $CaCl_2$ at 1 mg/mL concentration. Trypsin was added to the protein solution with a final trypsin:protein ratio at 1:200 (w/w), and the reaction mixture was incubated at 37 °C for 30 min. The hydrolysis of one tryptic peptide by endoproteinase Asp-N was carried out in 0.1 M potassium phosphate buffer at pH 7.8 and 37 °C for 0.5 h, with a protease:peptide ratio of 1:200 (w/w).

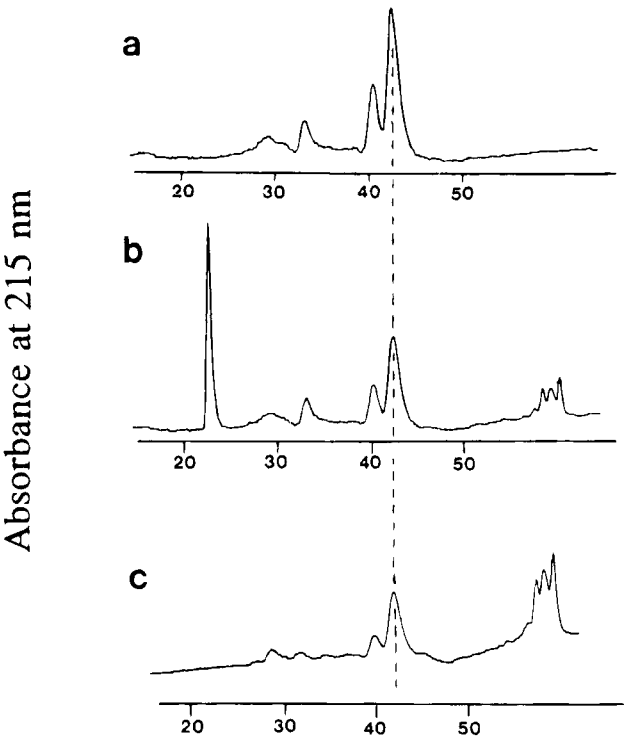

FIGURE 1: High-performance liquid chromatographic analyses of (a) commercial rabbit liver metallothionein, (b) commerical metallothionein treated with melphalan, and (c) Cd-reconstituted metallothionein treated with melphalan. Reaction conditions are discussed under Experimental Procedures.

Reversed phase HPLC was performed with a Shimadzu (Kyoto, Japan) SPD-6A pump system and C8 reversed phase columns (Aquapore RP-300, 4.6 × 250 mm, and 2.1 × 100 mm; Applied Biosystem, San Jose, CA). A typical flow rate was 1 mL/min (and 0.3 mL for the narrowbore column), and protein elution was monitored at 215 nm by a UV detector (Shimadzu). Solvents were as follows: A, 0.1% TFA/H_2O; B, 0.1% or 0.08% TFA in acetonitrile.

All ESI-MS experiments were performed on a Vestec (Houston, TX) electrospray source fitted to a Hewlett-Packard (Palo Alto, CA) 5988A quadrupole mass spectrometer, as described previously (Yu et al., 1993).

Fast atom bombardment mass spectrometry (FABMS) was performed on a JEOL HX110/HX110 tandem mass spectrometer (Tokyo, Japan), controlled by the DA 5000 or DA 7000 data system. The FAB source was operated at 6 kV, and the accelerating voltage was 10 kV. The resolution was set at 3000 for molecular weight determination. Peptides were dissolved in 0.1% TFA/H_2O solution, and 1 μL of sample was mixed with the matrix (9:1 v/v mixture of monothioglycerol and glycerol) on the sample probe. For tandem mass spectrometry (MS/MS), helium was used as the collision gas with 70% attenuation of the parent ions, and the collision cell was floated to 4 kV. Resolution was set at 1000 in all MS/MS experiments.

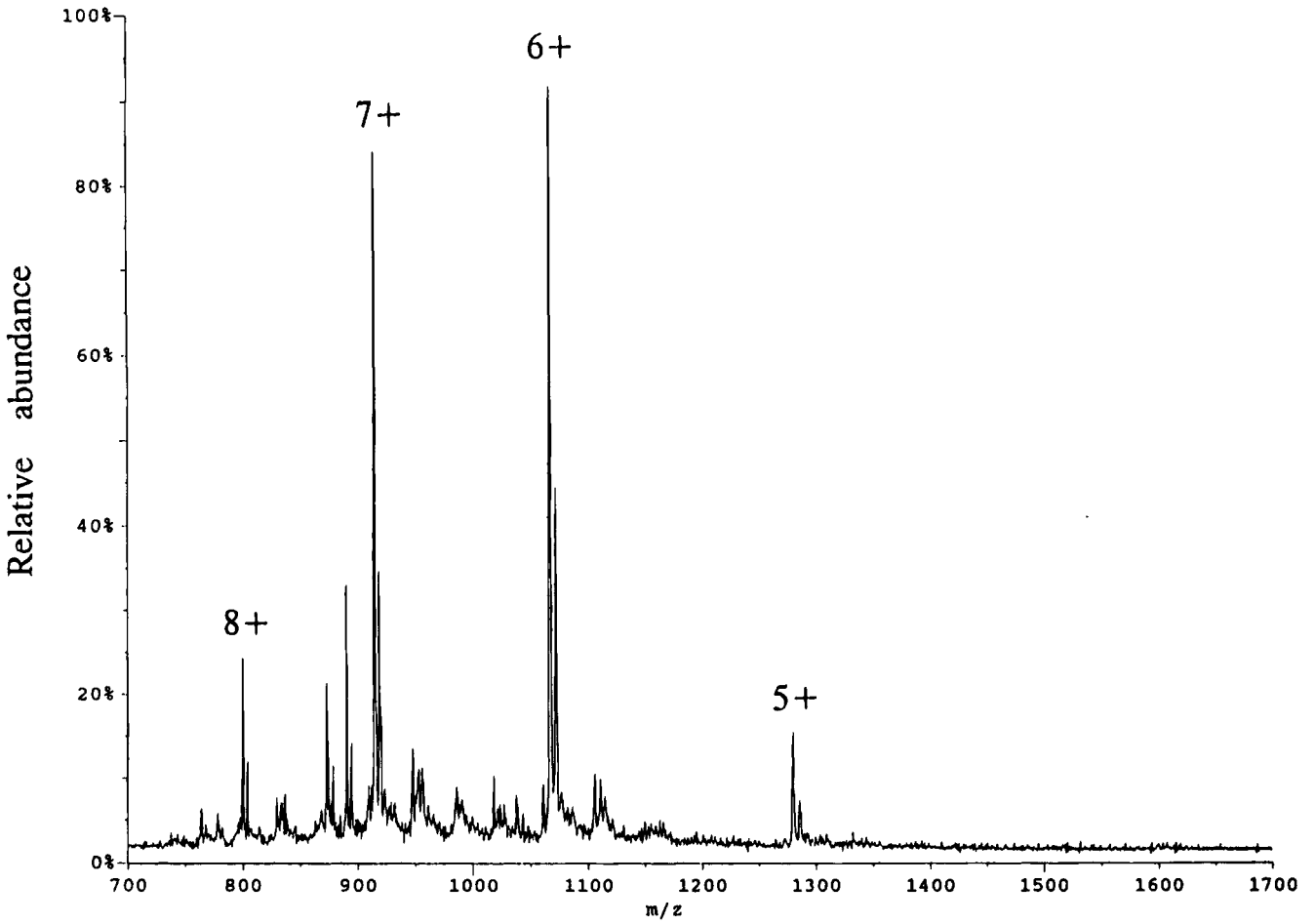

FIGURE 2: Electrospray ionization mass spectrum of melphalan-alkylated metallothioneins (HPLC-purified). The expected molecular masses for the major and minor alkylated proteins are 6394.0 and 6424.0 daltons. Observed molecular masses were 6393.5 and 6424.0 daltons, respectively.

RESULTS

Commercial MT was incubated with melphalan, and the reaction products were separated by reversed phase HPLC. As shown in Figure 1, several new peaks appeared at longer retention times than those of the unreacted MT subisoform peaks. These new peaks (57−63 min) were collected together and also in three fractions, and were analyzed by ESI-MS. Both the composite sample (Figure 2) and each of the three fractions were found to contain protein species with a molecular mass of 6393.5 daltons, indicating that they are 1:1 melphalan-alkylated MTs. The molecular mass of the major subisoform in commercial MT (MT-2a) is 6125.3 daltons, as reported previously (Yu et al., 1993). The net mass increase of the modified MT was 268.3 daltons, consistent with the addition of one melphalan moiety. The occurrence of multiple peaks in the chromatogram with the same molecular mass suggested that different residues were alkylated in the reaction. The minor component in the sample (MT-2a′, molecular mass 6155.2 daltons) was also found to form adduct(s) with melphalan, which coelected with the major products. The molecular ions of this alkylated minor subisoform are observed at each charge state in Figure 2, and the molecular mass was found to be 6424.0 daltons.

The yield of alkylation was estimated to be 20% (HPLC peak area) when MT and melphalan concentrations were 140 μM in the reaction. Increasing the concentration of both MT and melphalan to 560 μM yielded 48% 1:1 melphalan-alkylated MT without much formation of 2:1 or higher ratio adducts (determined by ESI-MS molecular weight measurements).

Reconstituted Cd_7-MT (180 μM) was also reacted with melphalan (560 μM) under similar conditions, and the alkylation products were analyzed by reversed phase HPLC and ESI-MS. An identical HPLC pattern of product peaks was observed as that found with native MT-2 (Figure 1c), with about a 38% yield of alkylation. The molecular mass of these adducts were also found to be 6393.5 daltons.

The melphalan-alkylated MT samples were methylated at free cysteine thiols and hydrolyzed by trypsin. The protein was completely digested within 30 min, and the tryptic peptides were mapped by reversed phase HPLC. Three new peaks were observed that were not present in the control digest of unalkylated MT (Figure 3). Each peak was collected separately and further analyzed by ESI-MS and FABMS. As listed in Table 1, the complete sequence of MT was mapped by the observation of all tryptic peptides expected. At the same time, three new species with molecular masses corresponding to 1:1 melphalan adducts were detected as well. The molecular mass of the peptide in fraction 1 was found to be 1121.7 daltons. This corresponds to the adduction of one melphalan to the peptide CAQGCICK (residues 44−51), which has an original molecular mass of 867.7 daltons. Similarly, HPLC fraction 2 was found to contain a peptide with a molecular mass of 1482.6 daltons, corresponding to the melphalan alkylation of peptide SCCSCCPPGCAK (residues 32−43). A peptide with a molecular mass of 2638 daltons was observed in

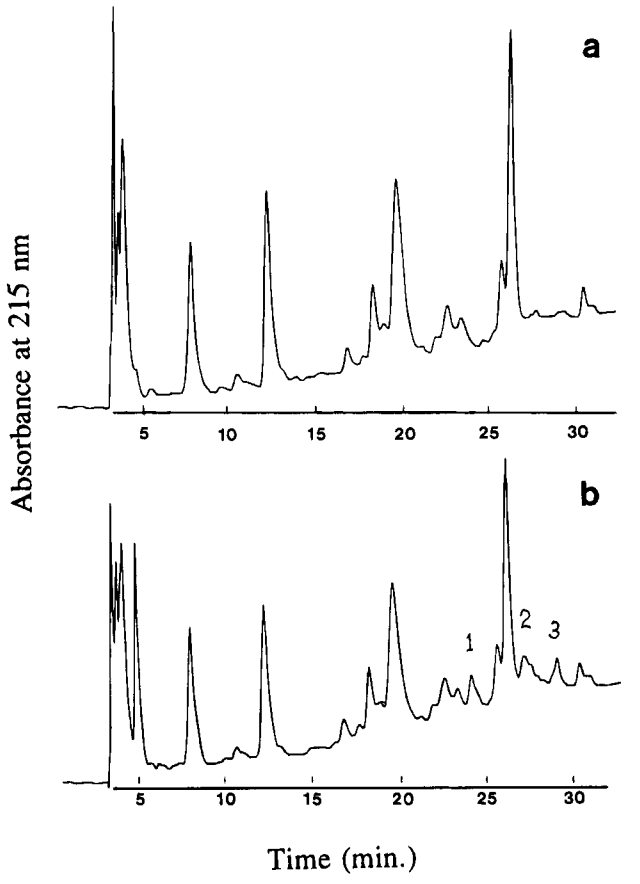

FIGURE 3: High-performance liquid chromatographic analyses of (a) trypsin-digested metallothionein and (b) trypsin-digested melphalan-alkylated metallothionein. Both protein samples were methylated on all free thiols before trypsin hydrolysis (see Experimental Procedures).

Table 1: Mapping of Cys-Methylated Metallothionein 2a[a] by Fast Atom Bombardment Mass Spectrometry

	monoiso M+H$^+$		alkylated peptides	
MT peptides	calculated	observed	calculated	observed
1−10[b]	1109.7	1110.2	1364.7	1364.2
11−22	1291.8	1292.2	1546.8	1546.2
23−25	335.1	335.7	589.1	
26−30	569.2	569.6	823.2	
26−31	697.3	697.6	951.3	
32−43	1228.3	1228.6	1482.3	1482.6
44−51	867.3	867.7	1121.3	1121.7
52−56	477.2	477.7	731.2	
57−61	528.1	528.5	782.1	

[a] AcMDPNCSCAAAGDSCTCANSCTCKACKCTSCKKSCCSCCPPGCAKCAQGCICKGASDKCSCCA. [b] Numbered according to Wagner et al. (1986).

HPLC fraction 3, suggesting the melphalan alkylation of peptide Ac-MDPNCSCAAAGDSCTCANSCTCK (residues 1−22). We have used the sequence numbering system conventional in this area (Wagner et al., 1986), in which the third A in this rabbit protein is A-8′.

Since each of these three tryptic peptides contains more than one cysteine, tandem mass spectrometry was required in order to locate the exact alkylation sites within the peptides. Peptide Ac-MDPNCSCAAAGDSCTCANSCTCK was further hydrolyzed by endoproteinase Asp-N. Two smaller peptides, Ac-MDPNCSCAAAG and DSCTCANSCTCK, were produced, and MS/MS experiments were performed.

The tandem mass spectrum of the melphalan-containing peptide SCCSCCPPGCAK is shown in Figure 4. Cleavages within the peptide backbone and the melphalan moiety were both observed. An extensive series of w ions is detected, generated by the cleavage of peptide at bonds between the amide nitrogens and the α-carbons, with additional loss of the side chain group of the cleaved residue (Biemann, 1990). The mass observed for the w_5 ion indicates that Cys 41 is methylated and not bonded to melphalan. Similarly, the masses of the w_8, w_{10}, and w_{11} ions identify methylation on Cys 37, Cys 36, and Cys 34, respectively. Modification of Cys 33 by melphalan is directly characterized by the mass of the y_{11} ions and the mass difference between y_{11} and w_{11} ions. Ions of mass 255 and 182 daltons are derived from the drug moiety itself. The series of peaks between *m/z* 1134 and 1241 reflects cleavages across each bond in the linker chain between the amide backbone and the mustard nitrogen, and locates the point of attachment in the mustard. Formation of a protonated disulfide bond is proposed with loss of the drug moiety on Cys 33 and the methyl group from adjacent Cys 34 (Budzikiewicz et al., 1967) to produce stable ions of mass 1999.5 daltons.

Using the same approach, we have identified Cys 48 as the only melphalan reaction site in peptide CAQGCICK. As shown in Figure 5, the w ion series is again important in this spectrum of ions formed by high-energy collisional activation (Biemann, 1990). Ions generated by cleavage at isoleucine (w_{3a} and w_{3b}) identify methylation on Cys 50. Methylation is confirmed on Cys 44 by the masses of a_2 and w_6 ions. Modification by melphalan is located at Cys 48 by the mass differences between w_3 ions and the w_4, x_5, and w_6 ions. Again, drug-derived ions occur with masses of 255 and 182 daltons, and cleavages in the linker chain contribute the major peak at *m/z* 805.5 and other intense peaks in the range *m/z* 790−880.

The mass spectrum (Figure 6) of peptide Ac-MDPNCSCAAAG supports partial alkylation by melphalan on both cysteine residues. It was necessary to convert the drug-alkylated and S-methylated peptide to a methyl triester in order to obtain sufficient ion current for collisional activation. With no strongly basic site in the peptide chain, the fragmentation pattern is different from those in the other spectra obtained in this study. The spectrum in Figure 6 contains internal fragment ions (Biemann, 1990), perhaps reflecting the presence of proline, low mass fragment ions derived from the drug moiety, and a number of sequence ions produced by cleavages in the polyamide backbone. Some of these have the same mass regardless of whether melphalan is attached to Cys 5 or to Cys 7; however, those formed by fragmentations between residues 5, 6, and 7 permit modifications of the two cysteines to be distinguished. Covalent attachment of melphalan to Cys 7 (as shown in the inset in Figure 6) is supported by b_5, w_6, x_6, z_6, and w_7 ions labeled in the spectrum and by two internal fragments, SCA and SCAAA. However, covalent attachment of melphalan to Cys 5 in a fraction of the peptide molecules in this sample is supported by a_6 and d_7 ions, whose designation is circled on the spectrum.

Peptide DSCTCANSCTCK was found to be partially alkylated by melphalan on Cys 13 and Cys 19, but not on Cys 15 and Cys 21. In the spectrum (Figure 7) of this lysine-containing peptide, a series of w ions and other sequence ions allow determination of the substituent (methyl group or melphalan) on each cysteine side chain. All sequence

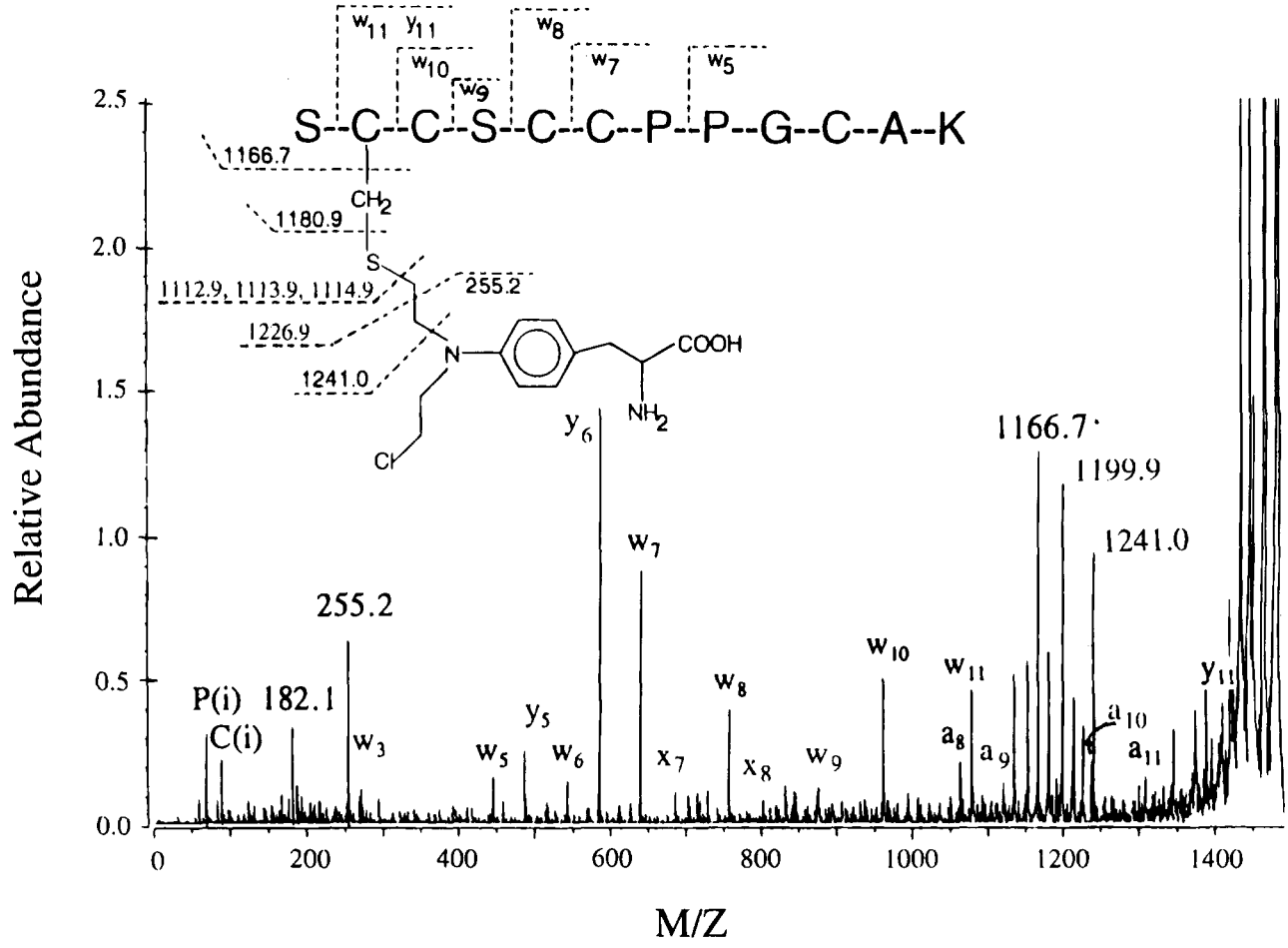

FIGURE 4: Tandem mass spectrum of peptide [SCCSCCPPGCAK]-melphalan, in which −SH groups have been converted to $-SCH_3$ groups. Major fragments are illustrated in the inset, which indicate alkylation at Cys 33.

ions in the spectrum define or are consistent with methylation of Cys 15 and Cys 21. The w ion series shown in the inset supports melphalan-alkylation at the first cysteine in the peptide (Cys 13) as shown in the inset. The ions with circled sequence letters are assigned to peptide molecules carrying the drug on Cys 19. Peaks between *m/z* 1200 and 1300 correspond to ions formed by cleavages at various places in the linker to melphalan. As in the spectrum in Figure 4, an abundant peak (*m/z* 1262.4) is assigned to ions formed by loss of the drug moiety and loss of a methyl group from sulfur, with formation of a protonated intrachain disulfide bond. Peaks labeled 255 and 182 are derived from melphalan.

Peak areas were estimated in HPLC separations detected at 260 nm (where melphalan has its maximum UV absorbance and apoMT absorbance is negligible). It was observed that the peptides in fractions 1, 2, and 3 represented 66%, 23%, and 11% of the total alkylation, respectively. Since no other peptides were found in the three HPLC fractions, it is concluded that alkylation at Cys 48 represents 66% of the total reaction, and that at Cys 33 accounts for 23% of the melphalan alkylation. The remaining 11% of the alkylation is distributed among cysteines 5, 7, 13, and 19.

The same three tryptic peptides that contain melphalan were identified in the reaction products of melphalan and reconstituted Cd_7-MT. The relative yields for peptides in fractions 1, 2, and 3 were estimated to be 65%, 22%, and 13%, respectively. It appears that the same reaction products were formed in similar relative amounts with the commercial mixed metal MT (Yu et al., 1993) and the reconstituted Cd_7-MT, as the experimental error is estimated to be around 5%. These peptides were not analyzed by tandem mass spectrometry due to the limited amounts of samples available.

In order to detect if structural changes occur in MT upon alkylation by melphalan, we have examined the trypsin susceptibility of the native MT and the melphalan-alkylated MT. Reversed phase HPLC analysis indicated that native MT is resistant toward trypsin hydrolysis and the protein was still intact after 24 h of incubation. Melphalan-alkylated MT was found to be partially cleaved into large fragments after 1 h of incubation with trypsin. Interestingly, cleavages within these two fragments were not observed to be significant after an additional 23 h of incubation. Analysis by FABMS revealed that the protein was cleaved in the middle of the polypeptide chain, at the two lysines (residues 30 and 31) in the hinge region. Major fragments were found to have molecular masses of 3020.2, 3124.5, and 3252.5 daltons, corresponding to the C-terminal domain, [31−61], and two N-terminal peptides, [1−30] and [1−31], respectively. We suggest that the 1:1 melphalan-alkylated MT became susceptible to trypsin proteo ′sis in the hinge of the polypeptide chain.

DISCUSSION

Since it was first reported that MT synthesis can be induced by exposure to the alkylating agent sodium iodoacetate (Kotsonis & Klaassen, 1979), it has been suggested that sulfhydryl groups in MT may act as binding sites for detoxification of electrophilic agents (Cagen & Klaassen,

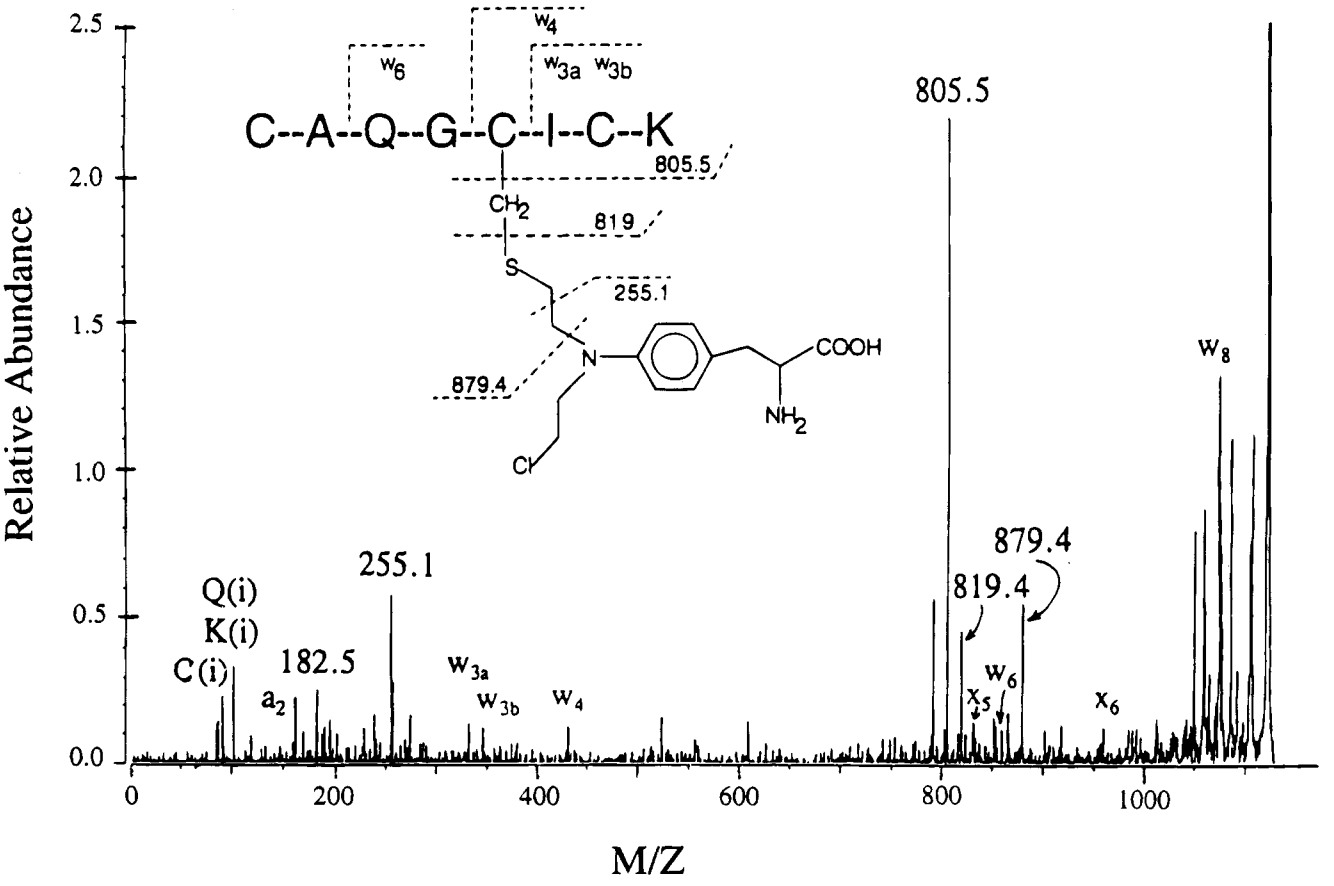

FIGURE 5: Tandem mass spectrum of peptide [CAQGCICK]-melphalan, in which −SH groups have been converted to $-SCH_3$ groups. Major fragments are illustrated in the inset, which indicate alkylation at Cys 48.

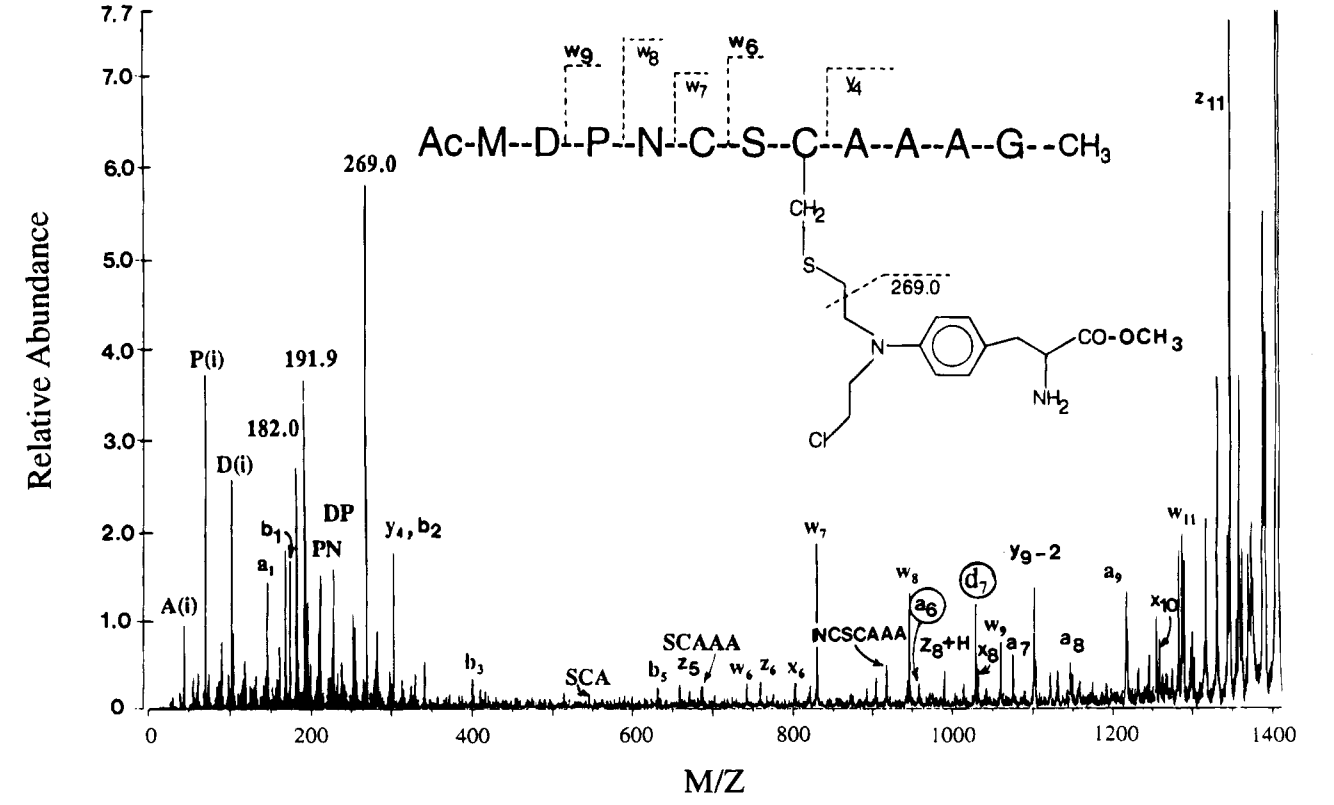

FIGURE 6: Tandem mass spectrum of peptide [Ac-MDPNCSCAAAG-CH_3]-melphalan, in which −SH groups have been converted to $-SCH_3$ groups and the methyl triester has been formed. Major ions are illustrated in the inset, which indicate alkylation at Cys 5. Circled sequence labels are assigned to molecules alkylated at Cys 7.

1980; Kelley et al., 1988). It is demonstrated in our study that direct covalent sequestration of the alkylating agent melphalan by MT occurs very rapidly and efficiently *in vitro* at near-physiological conditions. Melphalan concentration

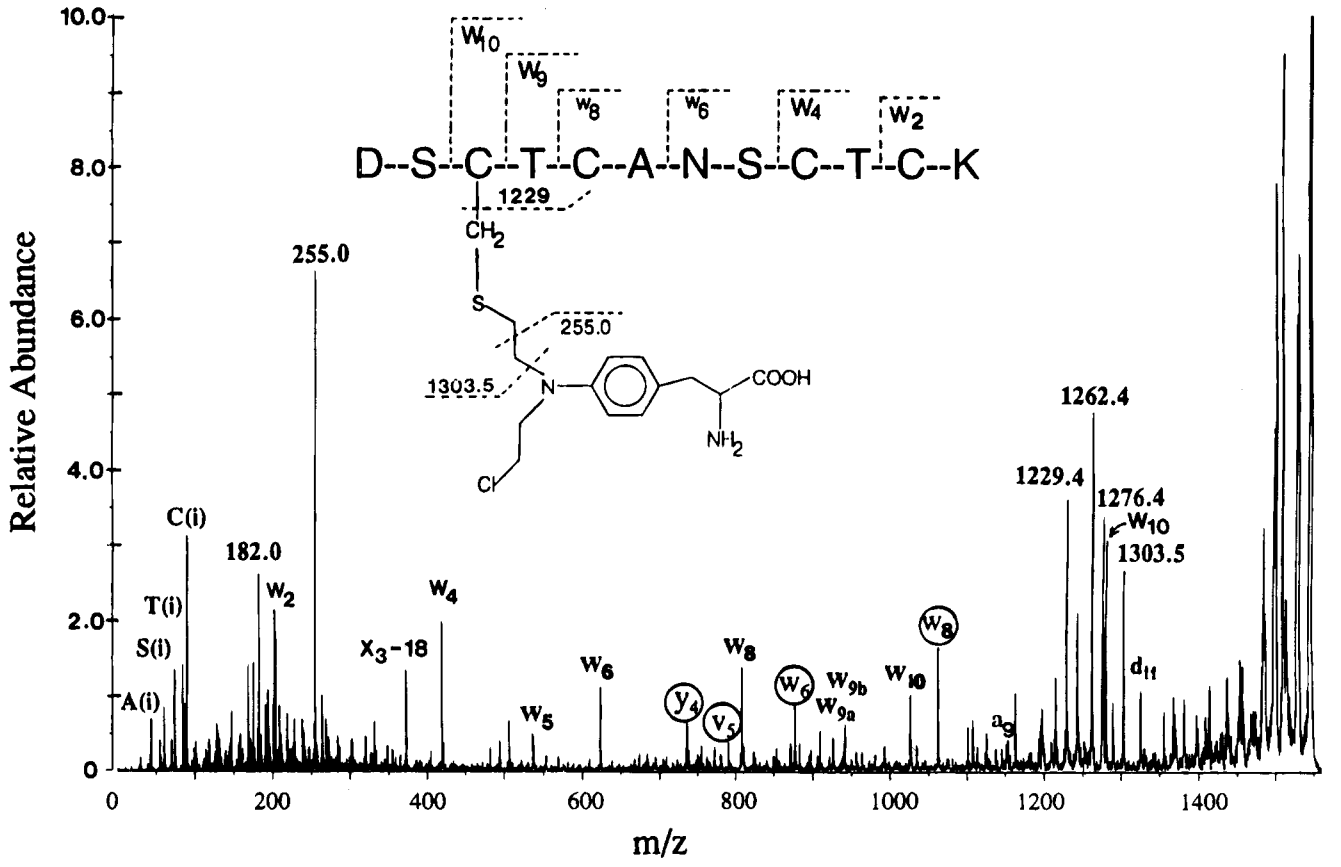

FIGURE 7: Tandem mass spectrum of peptide [DSCTCANSCTCK]-melphalan, in which −SH groups have been converted to $-SCH_3$ groups. Major ions are illustrated in the inset, which indicate alkylation at Cys 13. Circled sequence labels are assigned to molecules alkylated at Cys 19.

measured in plasma of human subjects has been reported to vary from 1 to 13 μM, depending on the route of administration (Colvin & Chabner, 1990), and the intracellular concentration is estimated to range from 5 to 130 μM (Bolton et al., 1993). It is observed that MT levels in liver or kidney tissues can vary from 1 to 10 μM at normal conditions to over 150 μM when induced by metals (Summer & Klein, 1991). Mouse cells transfected with the human MT-IIA gene displayed a more than 4-fold increase in resistance to *cis*-diamminedichloroplatinum, melphalan, and chlorambucil when the MT level was found to have increased more than 10-fold (Kelley et al., 1988). In tumor cells, synthesis of MT may be induced by activation of the Ha-*ras* oncogene (Schmidt & Hamer, 1986), or by prior exposure to therapeutical alkylating agents, interferons and steroids (Hamer, 1986). Thus, we suggest that intracellular levels of melphalan can be significantly reduced by MT covalent sequestration.

Glutathione (GSH) is a very important component in the cellular defense against electrophilic agents, and elevated GSH levels in cells have been associated with resistance to alkylating agents (Colvin et al., 1993). Accounting for 20 sulfhydryl groups per molecule, the sulfhydryl concentration due to MT could be comparable to that provided by glutathione (Allalunis-Turner et al., 1988) when the MT synthesis is induced. Therefore, we suggest that induced expression of MT may represent a significant contribution in the defense against alkylating agents. Furthermore, the extent of the nucleophilic reaction between melphalan and GSH is reduced close to 8-fold at pH 6.5 compared to that at pH 7.4 (Bolton et al., 1993). Since the pH in tumor cells is often lower than that of normal cells (Wike-Hooley et al., 1984), it will be interesting to determine the effect of pH on the MT sequestration of melphalan.

The selective pattern of melphalan alkylation detected in our study is highly intriguing. Two cysteines in the α-domain, Cys 48 and Cys 33, account for about 90% of the total alkylation. The remaining portion of the alkylation was found to occur among cysteines 5, 7, 13, and 19 in the β-domain. This is consistent with the report that reaction with the α-domain of MT represents the faster phase of the biphasic kinetics in the thiol−disulfide interchange reaction of MT and the aromatic disulfide 5,5′-dithiobis(2-nitrobenzoate) (Li et al., 1981; Savas et al., 1991). It has been suggested that terminal sulfurs are more negatively charged than bridging sulfurs (Robbins & Stout, 1991). In this study, five out of the six alkylated cysteines (Cys 5, 13, 19, 33, and 48) are terminal ones, consistent with the idea that they are more nucleophilic.

Solvent accessibilities of cysteine sulfurs in MT have been found to differ greatly in the crystal structure of rat liver MT (Robbins et al., 1991). Two solvent-exposed clefts were found to contain cysteines 5, 7, and 13 in the β-domain, and cysteines 37, 41, and 57 in the α-domain. It might have been expected that these cysteines would react more readily with melphalan. However, a number of these residues participate in intramolecular NH···S hydrogen bonds and may have strong hydrogen bond interactions with nearby water molecules as well. Presumably, hydrogen bonds to sulfur will stablize the deprotonated thiolate and reduce its reactivity toward electrophilic agents. The extent of exposure to solvent molecules as revealed in X-ray crystallographic studies (Robbins & Stout, 1991) may also correlate with the total accessibility of the four sulfurs bonded to each metal

in MT. Cysteines 33 and 48 chelate the most accessible metal in the α-domain, while in the β-domain cysteines 7 and 13 are bonded to the metal which has the largest total solvent accessibility in all seven metal sites.

However, other factors must be called on to explain the high preference for reaction with the kinetically less labile α-domain (Bernhard et al., 1986; Bernhard, 1991; Nielson & Winge, 1983). We propose that MT binds melphalan selectively and noncovalently prior to adventitious covalent alkylation of proximal cysteines. Electrostatic interactions between the positive aziridinium form to which melphalan converts spontaneously ($t_{1/2}$ = 40−44 min; Bolton et al., 1993) and negatively charged sites on MT and hydrophobic interactions involving the aromatic moiety of melphalan are two possible sources that may contribute to such binding. In addition, hydrogen bonding between the α-amino and/or carboxyl groups of melphalan and specific sites on MT was indicated by computer-supported "docking" studies to favor melphalan binding near the cadmium ion chelated by Cys 33 and Cys 48 (unpublished results).

Because MT was resistant to at least 24 h of trypsin hydrolysis under our conditions, we predict that no spontaneous loss of metals occurred during the 30 min alkylation reaction. However, the structural changes reflected by limited trypsin accessibility in the monoalkylated MT may be physiologically important. It is tempting to speculate that such alterations in the three-dimensional structure of MT could render to the alkylated MT different binding capabilities in its interaction with other cellular components, which in turn will change the level and pattern of defensive responses. It was recently discovered that such a system is utilized in the repair of aberrant DNA methylation by *Escherichia coli* Ada (Myers et al., 1993). Direct transfer of the methyl group from O^6-methylguanine to one of the four zinc-coordinated cysteines reveals a sequence-specific DNA binding activity in the N-terminal domain. This normally hidden activity enables Ada to activate the transcription of a methylation resistance gene. No role in gene regulation has been observed for MT as yet, although the protein has been found to accumulate in the nucleus (Banerjee et al., 1982; Chubatsu & Meneghini, 1993).

Subsequently, multiple alkylation by melphalan occurs (unpublished results). The metal ions bound in MT will eventually be released by multiple alkylation, and this can influence many biochemical processes in normal cellular functioning. Most significantly to the present study, the MT level itself will be affected, as increased metal concentration can be expected to induce intracellular MT synthesis. Thus, more protein will become available for continued detoxification of melphalan and other cross-resistant alkylating drugs.

Acquired drug resistance remains a challenge in cancer chemotherapy, as tumor cells become cross-resistant to many commonly used alkylating agents after long exposures. The genetic instability of many cancer cells can significantly potentiate the mutation frequency introduced by many mutagenic drugs used in cancer treatment, and produce resistant cells at a faster rate. Overexpression of enzymes and proteins involved in drug metabolism, drug transport, and repair of damaged targets are several common biochemical mechanisms in the development of drug resistance. Sequestration of the drug can significantly reduce or prevent interaction with target site. Our results are consistent with the hypothesis that MT plays a role in the development of resistance against several common therapeutic alkylating agents, as an alternative site for reactions with the alkylating agent melphalan.

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