Articles

A Binding Site for Chlorambucil on Metallothionein†

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ABSTRACT: It is of interest to test the hypothesis that induced metallothionein (MT) acts in acquired drug resistance by covalent sequestration. In this study MT was incubated *in vitro* with chlorambucil (CHB) under conditions where only 1:1 covalent adducts were formed. The proteolytic products of these adducts were analyzed by HPLC and mass spectrometry to reveal two major sites of modification. These were the sulfur atoms of cysteines 33 and 48, which cochelate the same metal atom in native MT. The time course of the reaction was followed using on-line electrospray ionization with a double-focusing mass spectrometer. These experiments showed that drug-modified MT binds seven metal ions, as does the unmodified protein. Molecular docking experiments showed that the selectivity of drug binding is influenced by the presence of the aziridinium ion in the drug structure and complementary charge densities in the protein structure.

The metallothioneins (MTs)[1] are a class of cysteine-rich, metal-binding proteins which occur ubiquitously in the cytosol of vertebrate and invertebrate cells. Mammalian MT contains 61 or 62 amino acid residues of which 20 are conserved cysteines (Kägi & Schäffer, 1988; Kägi, 1991). Native MT binds seven divalent d^{10} metal ions, usually Zn^{2+} and Cd^{2+}, and folds into a two-domain structure (Otvos & Armitage, 1980). Most commonly, amino acids 1−29 form the β-domain, which contains 9 cysteines and chelates 3 metal ions. The α-domain is composed of residues 33−61, contains 11 cysteines, and chelates 4 metal ions. It is known that the metal ions in each domain are bound and released in a highly cooperative manner (Nielson & Winge, 1983). Because MT is a small protein, the metal atoms are more exposed than those bound in other, larger metalloproteins (Otvos *et al.*, 1989). NMR studies have shown that the metal clusters are dynamic and that the metallothiolate bonds are continuously breaking and re-forming (Nettesheim *et al.*, 1985; Vašák *et al.*, 1986).

MT binds a variety of heavy metal ions and may function in heavy metal detoxification [reviewed by Hamer (1986)]. It is also active in zinc homeostasis, providing a pool of zinc that may be utilized as needed by a variety of cellular pathways (Li *et al.*, 1980). Zn^{2+} is required for basal and induced expression of MT (Minichiello *et al.*, 1994; Otsuka *et al.*, 1994), and its binding by apo-MT provides a feedback mechanism for MT expression (Nettesheim *et al.*, 1985; Otvos *et al.*, 1989). MT is induced at the transcriptional level *in vivo* by a variety of agents including heavy metal ions, hormones, oxidizing agents, and alkylating agents (Kotsonis & Klaassen, 1979; Hamer, 1986; Palmiter, 1987; Klaassen & Liu, 1991). It has been demonstrated that overexpression of MT confers cross-resistance to several therapeutic anticancer drugs, such as *cis*-diamminedichloroplatinum (II), melphalan, and CHB (Endresen *et al.*, 1983; Kelley *et al.*, 1988). The proposed mechanism is that increased amounts of MT provide increased intracellular drug sequestration, thus reducing the amount of alkylating agent reaching the target DNA. It is clear that MT functions to protect the cell from alkylating agents, presumably in parallel with other detoxification mechanisms that depend on induced proteins, for example, glutathione *S*-transferase (Hayes & Wolf, 1990; Colvin *et al.*, 1993). Despite extensive research on the activity of MT, no natural organic substrate has yet been identified.

Several laboratories have published studies in support of the hypothesis that the mechanism for the sequestration of alkylating agents by MT is covalent. Endresen and coworkers observed that the incubation of resistant cells with [^{14}C]-chlorambucil for 1 or 24 h caused 20−40% of the radioactivity to copurify with MT (Endresen *et al.*, 1983). No information was provided in that work regarding the structure of the MT−CHB complex(es). Recently, Yu *et al.* (1995) established that another nitrogen mustard drug (melphalan) forms covalent bonds to MT. One unexpected outcome of that study was that melphalan selectively alkylates two cysteines in the α-domain of the protein. These cysteines cochelate the same metal ion in the folded protein. It was proposed that the selective alkylation pattern was the result of selective association of the drug with MT and that consideration of the interactions might suggest structural elements of natural organic substrates for MT. The question was raised whether alkylation of thiolate groups leads to

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[1] Abbreviations: ACN, acetonitrile; CHB, chlorambucil; CHB*, dehydrochlorochlorambucil; CID, collision-induced dissociation; C−S, carbon−sulfur; ESI, electrospray ionization; FAB, fast atom bombardment; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; MT, metallothionein; MT-2a, rabbit metallothionein isoform 2a; MT-2a′, rabbit metallothionein isoform 2a′; TFA, trifluoroacetic acid.

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cooperative collapse of one or both of the MT domains, releasing metal ions to signal further induction of MT.

In the present study, the reaction of chlorambucil with MT was studied to test the mechanistic proposal for drug sequestration and to seek the basis for cross-resistance. Mass spectrometric techniques were used to identify the sites of modification on MT. On-line electrospray mass spectrometry experiments were applied to follow the course of the reaction and to monitor the stability of the MT metal clusters after alkylation by CHB. These studies have implications for understanding the basal functions of MT as well as modulating acquired drug resistance.

EXPERIMENTAL PROCEDURES

Materials. Chlorambucil and rabbit liver metallothionein 2 (lot 34H95161) were obtained from Sigma Chemical Co. (St. Louis, MO), and 5000 nominal molecular weight cutoff microcentrifuge filters from Millipore Corp. (Bedford, MA).

Isolation of Chlorambucil-Modified MT. The protein concentration of metallothionein samples was measured by dissolving an aliquot of the sample (approximately 10 μM) in HCl (100 mM) and measuring the absorbance at 220 nm and using the known molar absorbtivity of rabbit MT (48 200 mol^{-1} cm^{-1}) (Vašák *et al.*, 1987).

Rabbit liver MT-2 (1 mg/mL, 163 μM) was dissolved in potassium phosphate buffer (0.1 M, pH 7.4) and reacted with CHB (2:1 CHB:MT ratio, CHB dissolved in ACN at 10 mg/mL) for 30 min at 37 °C. Analytical studies of the reaction between CHB and MT employed a C8 reversed-phase column (Aquapore RP-300, 4.6 × 250 mm, Applied Biosystems, San Jose, CA) with a linear gradient program: 0 min, 10% B; 3 min, 20% B; 29 min, 21.3% B; 59 min, 28% B. The mobile phases for all HPLC separations were A (H_2O, 0.1% TFA), and B (ACN, 0.08% TFA) and the flow rate was 1 mL/min. For purification of the modified protein, the reaction mixture was separated immediately after alkylation by using a POROS R/H column (4.6 × 100 mm, Perseptive Biosystems, Framingham, MA). The flow rate was 2 mL/min, the column was equilibrated with 10% B, and the following steps in the mobile phase composition were made: 5 min, 20% B; 11 min, 30% B; 16 min, 80% B. The collected fractions were frozen and lyophilized. The fractions were dissolved in aqueous TFA (100 μL, 0.1%) and injected onto a short C4 reversed-phase cartridge (LC-304, Supelco Inc., Bellefonte, PA), washed with 10 mL of 10% B, and eluted with 80% B. The collected fractions were frozen and lyophilized.

The cysteine side chains of the apo-MT samples were methylated using a published procedure (Hunziker, 1991). The reaction was stopped by applying the mixture to a size exclusion column (PD-10, Pharmacia LKB Biotechnology Inc., Piscataway, NJ), which had been equilibrated with aqueous TFA (0.1%). The eluate was immediately loaded on a C4 reversed-phase cartridge and eluted as described above.

The cysteine-methylated MT (1 mg/mL) was dissolved in aqueous ammonium bicarbonate (0.1 M, pH 8.0) and digested with trypsin (1% by weight) at 37 °C for 30 min. The digest mixture was acidified using acetic acid (10% by volume) and injected onto a C8 reversed-phase HPLC column. The mobile phases used were as above with a flow rate of 1 mL/min and the following linear program: 0 min, 5% B; 18 min, 20% B; 28 min, 35% B; 35 min, 35% B; 40 min, 60% B. The collected fractions were frozen and lyophilized. The samples were dissolved in H_2O−0.1% TFA for mass spectrometric analysis.

Mass Spectrometry. Matrix-assisted laser desorption/ionization (MALDI) mass spectra were acquired using a Kratos MALDI III mass spectrometer (Manchester, U.K.). This instrument has a 337 nm nitrogen laser and may be run in either linear or reflectron mode. α-Cyano-4-hydroxycinnamic acid was used as the matrix, and the thin film sample preparation technique was employed (Vorm *et al.*, 1994). Each sample spot was washed with aqueous TFA (0.1%) to remove salts from the matrix/sample surface. The instrument was operated in reflectron mode for all samples, and external calibration was used. Under these conditions the mass accuracy was 0.1% or better.

High-energy collision-induced dissociation (CID) spectra were obtained using a JEOL Ltd. (Tokyo, Japan) HX110/HX110 tandem mass spectrometer in the positive FAB ionization mode. The operation of this instrument has been described (Sato *et al.*, 1987). Briefly, the precursor ions are selected using the first mass spectrometer so that only the ^{12}C species is passed into the collision cell. The precursor ions are then fragmented by collision with helium gas in a floated collision cell. A CID spectrum is generated by analyzing the fragment ions using the second mass spectrometer.

Electrospray mass spectra were collected using the first mass spectrometer of the JEOL HX110/HX110 instrument and an Analytica of Branford Inc. (Branford, CT) thermally assisted electrospray source. Solvent was infused into the source at 1 μL/min using a syringe pump, and the interfacing capillary was heated to 120 °C. The resolution was set to 500 and the mass accuracy was 0.03% or better. Apo-MT (10 μM) was dissolved in H_2O−methanol−acetic acid (49:49:2) and pumped into the source. For dynamic analysis of the reaction between MT and CHB, the MT sample was desalted (but not denatured) using the centrifugal 5000 nominal molecular weight cutoff filter with three washes of H_2O. The reaction was monitored by mixing CHB and MT in 2:1 ratio using the same MT concentration (1 mg/mL) as in off-line studies (*vide supra*) but substituting ammonium acetate (10 mM, pH 7.4) for potassium phosphate buffer. The reaction was monitored continuously for 60 min at room temperature.

Molecular Dynamics Calculations. All calculations/simulations were executed using the QUANTA/CHARMm program (version 4.0) supplied by Molecular Simulations Inc. (Waltham, MA) using the crystal structure of rat MT (Robbins *et al.*, 1991) from the Brookhaven Protein Data Bank. CHB was modeled in its aziridinium form with a deprotonated carboxylic acid tail. After energy minimization, the lowest potential energy form was a structure with the tail curled toward the aziridinium group. CHB was then solid-docked with MT so that the aziridinium ring carbons were positioned as close as possible to each of the sulfur atoms of nine of the cysteine residues. At each of those docked positions, CHB was subjected to dynamics simulations (heating, 500 K, 1 ps; equilibrium, 500 K, 1 ps; simulation, 500 K, 10 ps) and the lowest energy form given by simulation was minimized (steepest descents, 5000 steps). Distance constraints between the closest aziridinium carbon to the cysteine sulfur were set at 3.0 Å during all steps. The

energies of the final minimized form of CHB were recorded. The aziridinium center was then moved along the carbon−sulfur (C−S) axis, and the energies were calculated at various points. The distances at which minimum energy was calculated were then compared for the cysteines tested to determine the most favorable sites of CHB alkylation on MT.

RESULTS

In preliminary studies the reaction between MT and CHB was monitored from the appearance of peaks that absorbed at 260 nm in the reversed-phase HPLC chromatogram of the reaction mixture. Unmodified MT lacks a 260 nm chromophore, and thus the new peak was tentatively identified as CHB-modified MT. Unmodified MT eluted at 51 min, and the CHB-modified protein eluted at 72 min. Using the reaction conditions described in the next paragraph, the chromatographic peak heights of reacted and unreacted MT indicated that 38% of the MT had been modified by CHB.

Rabbit liver MT-2 was reacted with CHB (2:1 CHB:MT ratio) for 30 min at 37 °C. A POROS R/H HPLC column was used to isolate CHB-modified MT, as described in Experimental Procedures. The molecular weight of the isolated material was determined using ESI-MS. Two products were detected with molecular weight values of 6391.0 and 6354.5. These values are in good agreement with the calculated molecular weight of 6392.93 for MT that has been modified with one CHB molecule and 6356.47 for the same molecule after the loss of 36.46, corresponding to one molecule of HCl, respectively. Hereafter, CHB* will be used to denote CHB that has lost a molecule of HCl.

The CHB-modified MT was methylated to prevent further reaction of the cysteine side chains. The molecular weight of methylated CHB-MT was determined to be 6623.2 by ESI-MS, a value that is in good agreement with the theoretical weight of 6622.99 for methylated MT that has a CHB* modifying group.

Methylated CHB-modified MT was digested with trypsin, and the digest mixture was fractionated by RP-HPLC. The tryptic peptides were analyzed by MALDI-TOF MS, and the protonated peptide *m/z* values were used to generate a tryptic map of MT. The MT peptides accounted for the entire sequence and were identified from these *m/z* values as shown in Table 1. Several *m/z* values are consistent with those expected for methylated, CHB- or CHB*-modified tryptic peptides of MT-2a. All observed masses are within 0.1% of the corresponding calculated values.

High-energy CID MS was used to determine the sequence and sites of alkylation of the modified peptides. MT was incubated with CHB for 10 min for this experiment, resulting in the detection of a greater abundance of peptides with the CHB rather than the CHB* modifying group. CHB modification at Cys-33 and Cys-48 was determined from CID spectra of peptides containing these residues. The sites of modification for the minor products were not determined.

CID spectra of the two modified peptides found in greatest abundance are shown in Figures 1 and 2. The largest peaks in both spectra are produced by fragmentation of the CHB moiety. These ions are useful to confirm that the precursor ion corresponds to a peptide modified with CHB. The ions labeled 1*−5* are produced from fragmentation that results in loss of part or all of the CHB group as neutral fragments

Table 1: Rabbit Metallothionein Tryptic Peptides Identified from Mass Spectra[a]

MT peptide[b]	*m/z* observed	*m/z* calculated
1−22	2384.3	2383.78
1−22 + CHB*	2602.0	2600.90
23−31 + CHB*	1232.2	1231.44
26−51 + CHB*	2977.0	2974.68
31−43	1357.9	1357.75
32−43	1229.7	1229.58
32−43 + CHB*	1447.0	1446.70
32−51 + CHB	2330.6	2332.26
44−51 + CHB*	1085.1	1085.25
52−61	987.0	987.16

[a] MALDI-TOF mass spectra were acquired for HPLC-purified tryptic peptides produced from CHB-modified MT. [b] The sequence used for rabbit MT-2a is MDPNCSCAA**A**AG[10]DSCTCANSCT[20]CKACKCTSCK[30]KSCCSCCPPG[40]CAKCAQGCIC[50]KGASDKCSCC[60]A (Kägi & Kojima, 1987). The sequence enumeration is the same as for rat MT-2. This entails that the insertion site, alanine in position 9, is labeled as 8′, thus keeping the same numbering of all residues common to the two sequences (Vašák *et al.*, 1987).

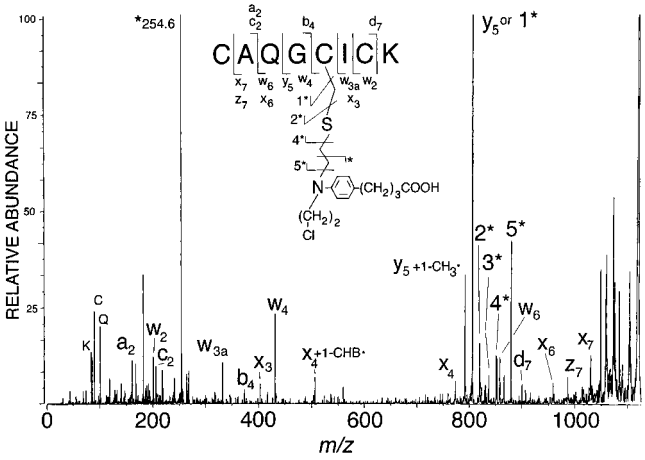

FIGURE 1: Collision-induced dissociation mass spectrum of rabbit metallothionein peptide 44−51 (CAQGCICK) modified by chlorambucil at position 48. Protonated molecular ion mass-to-charge ratio equals 1120.6 Da. The ions labeled with an asterisk are produced by the fragmentations shown in the inset. Cysteines at positions 44 and 50 have been methylated. The peptide backbone fragmentations are denoted using standard notation (Biemann, 1988; Roepstorff & Fohlman, 1984).

as shown in the inset of Figure 1. The ion labeled 3* results from the formation of a protonated intrachain disulfide bond accompanied by losses of the CHB moiety and a methyl group (Budzikiewicz *et al.*, 1967; Yu *et al.*, 1995).

In Figure 1, the *m/z* value of the precursor ion is 1120.6 Da, consistent with the protonated, CHB-modified MT peptide 44−51 (CAQGCICK),[2] expected *m/z* = 1120.97 Da. The presence of w_2, w_{3a}, and w_4 ions, all lacking the CHB group, eliminates the cysteine residue at position 50 as a site of modification. The presence of the w_6, d_7, x_6, z_7, and x_7 ions, all of which include the CHB group in their structure, supports the conclusion that Cys-48 has been modified. The $x_4 + 1 - CHB\cdot$ and $y_5 + 1 - CH_3\cdot$ ions are of a type that has recently been described (Downard & Biemann, 1995) and are typically formed from CHB-modified peptides during high-energy CID (Zaia & Fenselau, 1995). Further confir-

[2] The three-letter amino acid code is used in the text when discussing positions in the metallothionien sequence. The single-letter code is used when a peptide or protein sequence is given.

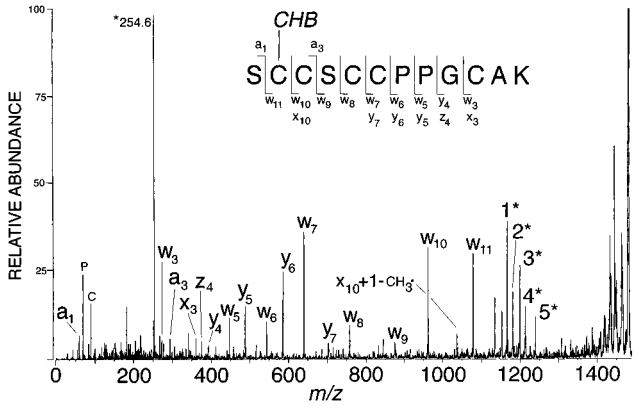

FIGURE 2: Collision-induced dissociation mass spectrum of metallothionein peptide 32−43 (SCCSCCPPGCAK) which has been modified with a CHB group at position 33. Protonated molecular ion mass-to-charge ratio equals 1481.2 Da. The ions labeled with an asterisk are produced by fragmentations similar to those shown in Figure 1. Cysteines at positions 34, 36, 37, and 41 are methylated.

mation of the alkylation site comes from CID spectra (not shown) of the peptide modified by CHB* and by CHB in which the chlorine atom has been displaced by CH_3CH_2S- (Jiang, 1994).

The CID spectrum shown in Figure 2 was produced from a 1481.2 Da precursor ion. This mass is consistent with protonated MT peptide 32−43 (SCCSCCPPGCAK) modified with CHB, expected *m/z* = 1482.05 Da. The fragment ions labeled w_5, y_5, w_6, y_6, w_7, w_8, w_{10}, and w_{11} have masses that are consistent with methylated cysteines at positions 34, 36, 37, and 41. These ions indicate that Cys-33 is the site of CHB modification.

The relative extents of CHB modification at different sites on the MT chain were estimated by comparing the peak heights in the chromatogram of the tryptic digest products detected at 260 nm. Since incomplete tryptic cleavages resulted in peptides of different length that contained the same modified cysteine, the heights of the peptides containing a common modified cysteine were added for the purposes of quantitation. Some of the modified peptides were detected with both CHB and CHB* modifying groups, and absorbance peaks containing peptides with the same modified cysteine but differing by this loss of HCl were also summed. Modified peptide 1−22 accounts for 5% of the total modified MT, modified peptide 23−31 for 3%, alkylation at Cys-33 for 26%, and alkylation at Cys-48 for 66%.

The reaction between MT and CHB was directly monitored using ESI-MS. The reaction was conducted using the same concentrations of reactants as above, substituting ammonium acetate (10 mM, pH 7.4) for potassium phosphate. For practical reasons the reaction was conducted at room temperature, and data acquisition was started at $t = 3$ min. Figure 3 compares *m/z* range 1500−1900 at (a) $t = 3$ min and (b) $t = 60$ min. The masses calculated for the metallated MT species are within 0.10% of the observed values shown in the figure. Spectra at the beginning of the time course showed an abundant group of ions with *m/z* values corresponding to the 4+ charge states of Cd_4Zn_3-MT, Cd_5Zn_2-MT, Cd_6Zn_1-MT, and Cd_7-MT in the approximate ratios of 1:2:2:1. In Figure 3b, the masses of the MT ions have shifted by the amount expected from modification by one CHB molecule. The metal compositions are identical to those at the beginning of the reaction. Note that

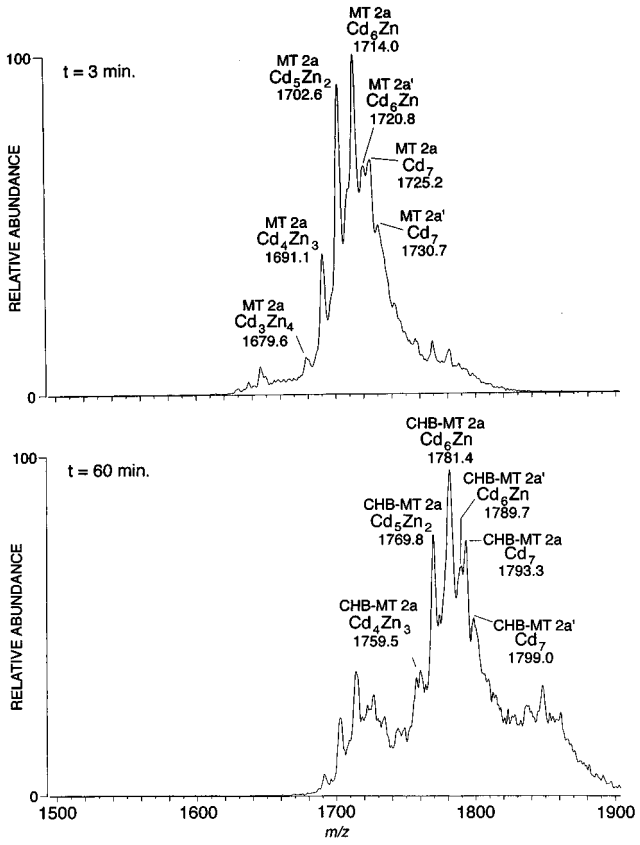

FIGURE 3: ESI MS spectra obtained by monitoring the reaction between MT and CHB on line: (a) $t = 3$ min; (b) $t = 60$ min. All ions are in the 4+ charge state and correspond to rabbit metallothionein 2a or 2a′ with various cadmium/zinc metal ion compositions.

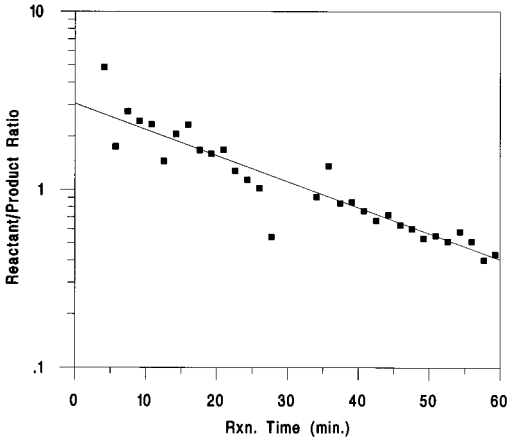

FIGURE 4: Product formation for the reaction between rabbit metallothionein and chlorambucil monitored on-line by electrospray ionization mass spectrometry. Each point is the quotient of the reactant and product ion abundances at a given time.

the MT mixture contains a small percentage of MT-2a′ for which Thr is substituted for Ala-8′ (see footnote, Table 1), resulting in a positive shift in mass of 30.01 Da (7.50 Da for the 4+ ions) (Hunziker *et al.*, 1995). Figure 4 shows the disappearance of unmodified MT over time. The abundances of the multiple metal compositions and isoforms were summed for each time point in order to calculate the reactant/product quotient.

DISCUSSION

Dehydrochlorination of CHB is caused by the conditions used to purify the modified peptides. This can be seen from

the fact that the on-line monitoring experiment showed only modification of MT by intact CHB. Since the half-life of CHB in aqueous solution in the pH range of 7−9 is 16 min (Chatterji *et al.*, 1982), detection of peptides modified by CHB* (dehydrochloro-CHB) under the experimental conditions is not surprising. No evidence was detected for cross-linking two sulfur atoms.

Spectra shown in Figure 3 were acquired by continuously scanning the mass spectrometer as a reaction mixture containing MT and CHB was infused from a syringe pump. The spectral quality was sufficient to allow the separation of different metal compositions of MT. Formation of apo-MT (m/z = 1532.23 for the 4+ charge state) was not detected during the 60 min reaction period, although its formation in a control reaction between MT and EDTA was readily monitored (data not shown). In the control experiment both holo- and apo-MT could be detected concurrently using the same instrumental tuning procedure used to follow the CHB reaction. If modified MT had released metal ions from either domain, the new species would also have been detected by ESI-MS (Yu *et al.*, 1993).

The conversion of reactants to products over 60 min is shown in Figure 4. This is the first time that ESI-MS has been used to monitor directly a reaction of a metal-binding protein and clearly shows the potential of this type of experiment. Although not a rigorous kinetic study, the linearity of the curve in Figure 4 as a function of time suggests that alkylation proceeds as a first-order reaction. This would be consistent with alkylation occurring as the slow step following formation of a drug/protein complex. Kinetic studies are the subject of further research in this laboratory.

Iodoacetamide (Bernhard *et al.*, 1986) and *p*-(hydroxymercuri)benzoate (Templeton *et al.*, 1986) have been found to react preferentially with the β-domain while 5,5′-dithiobis-(2-nitrobenzoic acid) (Savas *et al.*, 1991), melphalan (Yu *et al.*, 1995), and CHB (present work) have been shown to react preferentially with the α-domain. An associative binding mechanism is proposed to explain the positional selectivity defined experimentally for the latter two chemotherapeutic agents.

Molecular dynamics and solid-docking simulations were performed in order to identify some of the structural features of the MT molecule that might be important in the selective binding with CHB. These experiments were performed for the approach of CHB to the sulfur atoms of cysteines 5, 7, 13, 29, 33, 37, 41, 48, and 57, representing both the α- and β-domain. The rat MT-2 X-ray crystal structure (Robbins *et al.*, 1991) was used, because it is the only one available in which the intradomain geometry is known. Although the experiments presented here used rabbit MT-2a, comparison of the molecular dynamics results is considered to be valid because these two mammalian MTs are known to be isostructural (Vašák *et al.*, 1987).

Following dynamics simulations and energy minimization (see Experimental Procedures) the electrostatic, Lennard-Jones, and total potential energies were calculated as functions of aziridinium C−S atom distances for the approach of CHB to a given cysteine sulfur atom. Results for the nine cysteines are shown in Table 2. Cys-48 was the site for which the lowest potential energy was calculated and at the shortest C−S distance. At this site total electrostatic energy was the most favorable among the sites tested. The Lennard-Jones term was also the most favorable, allowing a C−S distance of less than 3.1 Å before repulsive overlaps begin to occur. The drug molecule uncurled as it interacted with the protein. Steric hindrance appears to prevent cross-linking by the second reactive arm of the mustard. Protein breathing in solution is expected to permit the closer approach required for bond formation.

Table 2: Relative Binding Energies for Chlorambucil and Metallothionein at Nine Sulfur Atoms

position	potential energy[a]	Lennard-Jones energy[a]	electrostatic energy[a]	C−S distance (minimum)[b]
Cys-5	−48.4	−10.3	−137.1	6.9
Cys-7	−136.3	−14.4	−220.5	8.5
Cys-13	−21.0	−6.7	−112.8	6.5
Cys-29	−96.0	−13.3	−183.9	14.1
Cys-33	−140.7	−14.1	−228.7	3.8
Cys-37	−117.1	−6.0	−216.0	4.4
Cys-41	−103.0	−9.1	−196.5	5.5
Cys-48	−153.7	−16.2	−239.3	3.2
Cys-57	−114.5	−6.0	−212.3	4.7

[a] kcal/mol. [b] Å.

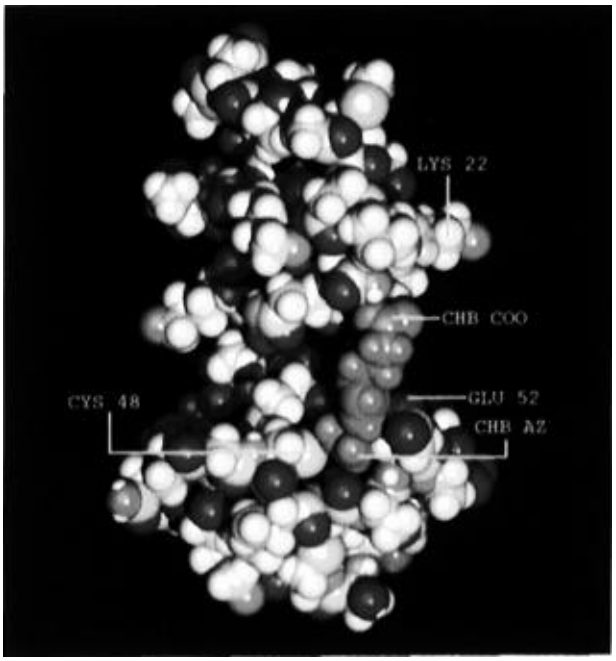

FIGURE 5: Model of chlorambucil bound to metallothionein so that reaction may occur with cysteine 48. This model corresponds to the lowest energy point for the CHB approach to cysteine 48 in Table 2. CHB AZ = chlorambucil aziridinium moiety.

For these simulations the protein backbone and side chains were constrained to the published (Robbins *et al.*, 1991) geometry. This choice was made because the appropriate molecular modeling parameters are not yet defined in the QUANTA/CHARMm energy minimization routines. The geometries of the metallothiolate clusters were altered in the minimized structure, a change that was judged to be unacceptable because of the weight of evidence supporting the published geometry (Frey *et al.*, 1985; Schultze *et al.*, 1988; Robbins *et al.*, 1991). The consistency between the theoretical and the observed results supports the validity of the method.

Figure 5 shows a model of CHB bound to MT such that alkylation of Cys-48 can occur. The CHB carboxylate is oriented toward the ammonium group in the side chain of Lys-22. The sulfur atoms of Cys-48 and Cys-33 (to the left

of Cys-48) are visible and accessible to the aziridinium electrophile in the interdomain gap. While the carboxylate group of Glu-52 in the rat structure seems positioned to interact with the aziridinium cation of the drug, this side chain cannot influence the observed chemical reactivity with rabbit MT because glycine is present at position 52 in the rabbit sequence. When the C−S energy calculations were repeated for Cys-48 and Cys-33 with glycine substituted for Glu-52 in the rat MT structure, the values for energy *vs* distance were within 4 kcal/mol of those in Table 2. No special role could be defined for the aromatic ring.

Conclusions. The results of this modeling study are consistent with the hypothesis that the selective alkylation pattern reflects selective binding followed by adventitious chemical reactions. The fact that both CHB and MEL react predominantly with Cys-48 and Cys-33 indicates that common structural features of these drug molecules associate with MT in a specific manner. Molecular dynamics calculations indicate that a binding pocket is formed within the region bordered by the two domains and the linking region of MT. Within this region, the CHB aziridinium ion appears to be attracted by the relatively strong negative character surrounding Cys-48 and Cys-33. This study and that with melphalan (Yu *et al.*, 1995) suggests that structural requirements for as yet unidentified endogenous substrates include a strong cationic center. The investigation of additional structural requirements for binding at this site is continuing with other therapeutic mustards.

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