Metallothionein (MT) releases zinc under oxidative stress conditions in cultured cells. The change in the MT molecule after zinc release in vivo is unknown although in vitro studies have identified MT disulfide bond formation. The present study was undertaken to test the hypothesis that MT disulfide bond formation occurs in vivo. A cardiac-specific MT-overexpressing transgenic mouse model was used. Mice were administered saline as a control or doxorubicin (20 mg/kg), which is an effective anticancer drug but with severe cardiac toxicity at least partially because of the generation of reactive oxygen species. A differential alklylation of cysteine residues in MT of the heart extracts was performed. Free and metal-bound cysteines were first trapped by N-ethylmaleimide and the disulfide bonds were reduced by dithiorthioetol followed by alklylation with radiolaabeled iodoacetamide. Analyses of the differentially alklylated MTs in the heart extract by high preformance liquid chromatography, SDS-PAGE, Western blot, and mass spectrometry revealed that disulfide bonds were present in MT in vivo under both physiological and oxidative stress conditions. More disulfide bonds were found in MT under the oxidative stress conditions. The MT disulfide bonds were likely intramolecular and both α- and β-domains were involved in the disulfide bond formation, although the α-domain appeared to be more easily oxidized than the β-domain. The results suggest that under physiological conditions, the formation of MT disulfide bonds is involved in the regulation of zinc homeostasis. Additional zinc release from MT under oxidative stress conditions is accompanied by more MT disulfide bond formation.

Previous studies have shown that metallothionein (MT) functions in cardiac protection against oxidative damage (1–4). The mechanism of the antioxidant action of MT remains elusive. It has been shown in vitro that MT directly interacts with reactive oxygen and nitrogen species to prevent oxidative and nitrosative stress. In vitro studies have shown that the interaction between MT and nitric oxide (NO) results in the release of 3 metals from the β-domain, leaving the α-domain metal binding unchanged (11), whereas other studies with synthetic zinc-reconstituted domain peptides demonstrate that S-nitrosothiols indeed release zinc from both the α- and the β-domains of MT (12). That MT is involved in zinc mobilization by NO in cultured cells has been demonstrated in several studies. Treatment of lung fibroblasts isolated from wild-type and MT-null (MT-KO) mutant mice with S-nitrosoyacysteine caused a differential zinc mobilization response. Specifically, an increase in intracellular labile zinc, as detected by a zinc-specific fluorophore Zinquin, was observed in the wild-type but not in the MT-KO fibroblasts (13). Furthermore, an elegant study by Pearce et al. (14) has shown that green fluorescent protein-modified MT undergoes conformational changes in the presence of NO in cultured pulmonary artery endothelial cells leading to the release of metals from MT.

MT becomes oxidized under conditions of oxidative and nitrosative stress in vitro (11, 13, 15–18). MT nitrosylation leads to disulfide bond formation in the β-domain (11). Upon storage under aerobic conditions, MT becomes dimerized through a disulfide bridge between two cysteine residues in the C-terminal α-domain (19). Although the Cu₄ cluster in the β-domain of Cu₄Zn₄-MT-3 was stable to air oxidation, the Zn₄ cluster in the α-domain was found to be air sensitive resulting in the release of one zinc ion, presumably accompanied by the formation of disulfide bonds. This process can be reversed under reducing conditions indicating the redox sensitivity of the Zn₄ cluster (20). An important question that remains to be answered is whether the formation of MT disulfide bonds occurs in vivo.

It is thus important to determine whether MT becomes oxidized in vivo to elucidate the mechanism of action of MT. Although experiments aimed at estimating the level of oxidized MT in weanling rat liver cytosol have been carried out, no precautions were taken to exclude the oxidation of MT during sample processing (21). In the present study, a cardiac-specific MT-overexpressing transgenic (MT-TG) mouse model was employed (1), which has proven to be a valuable tool in dissecting the role of MT in cardiac protection from...
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oxidative stress induced by an anticancer drug doxorubicin (DOX). In these mice, MT was overexpressed solely in cardiomyocytes and all other major antioxidants, including GSH, GSHpx, superoxide dismutase, and catalase, were not changed (1). The results obtained from the present study demonstrate that MT undergoes disulfide bond formation under physiological conditions in the hearts of MT-TG mice. In response to DOX-induced oxidative stress, MT becomes more oxidized, recruiting more cysteine clusters to form additional disulfide bonds.

MATERIALS AND METHODS

Reagents—N-Ethylmaleimide (NEM), iodoacetamide (IAA), dithiothreitol (DTT), protease inhibitors, and doxorubicin hydrochloride (adriamycin, DOX) were purchased from Sigma. Iodo-[14C]acetamide ([14C]IAA) and PD-10 desalting column were purchased from Amersham Biosciences. Other reagents were purchased from Sigma, or otherwise stated.

Animals and DOX Treatment—The cardiac-specific MT-TG mice produced previously (1) were used in the present study. The MT-TG mice express human MT-IIa specifically in the heart. Mice were housed and 50–95% B for 15 min at a flow rate of 1.0 ml/min. All fractions were dissolved in physiological saline by a single intraperitoneal injection at a volume of saline. Animals were sacrificed 24 h after the treatment, and the supernatants containing MT were loaded onto PD-10 desalting columns. The eluates were centrifuged at 15,000 g for 15 min, and the supernatants containing MT were loaded onto PD-10 desalting columns. The eluates were treated with 5 mM DTT or phosphate-buffered saline for 45 min at 37 °C. All samples were then treated with [14C]IAA (5.2 dpm/pmol of a total 0.5 mM) and incubated overnight at room temperature in the dark.

High Performance Liquid Chromatography Separation (HPLC)—The samples, prepared as described above, were concentrated with a Microcon YM-3 (Millipore, Bedford, MA) and loaded onto a reversed-phase column (3.9 × 150 mm, Delta Pak 5u C4, Waters, Milford, MA) equilibrated with eluent A (5% acetonitrile, water, 0.1% trifluoroacetic acid). Analysis was performed on a Beckman HPLC system (Beckman) consisting of a model 126 Programmable Solvent Module and a model 166 Programmable detector. Solvent programming, data collection, and analysis were accomplished using Beckman Nouveau Gold software. Proteins were eluted with a gradient of 0–25% eluent B (95% acetonitrile, water, 0.1% trifluoroacetic acid) for 10 min, 25–50% B for 50 min, and 50–95% B for 15 min at a flow rate of 1.0 ml/min. All fractions were collected and subjected to scintillation counting for identifying [14C]carbamidomethylated fractions and to immunoassay for MT detection.

SDS-PAGE, Western Blot, and Autoradiography—An aliquot of samples, prepared as described above, or dried HPLC fractions were dissolved in a non-reducing Western blot sample buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.05% bromphenol blue), and were separated on a 15% polyacrylamide gel. A Seefblue Prestained Protein Standard (Invitrogen) was used as molecular weight markers. Gels were electroblotted to polyvinylidene difluoride membranes and probed for MT using a mouse anti-MT monoclonal antibody (Dako Cytomation, Carpinteria, CA) as primary antibody and horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences) as secondary antibody, followed by chemiluminescent detection (ECL, Amersham). SDS-PAGE gels, performed in parallel, were electroblotted onto nitrocellulose membranes. Autoradiography was performed by exposure of the membranes to Kodak BIO-MS films at −80 °C for 7 days.

Trypsin Digestion—Bands on SDS-PAGE were excised from the gel, and the proteins were digested with 25 ng/μl sequencing grade modified trypsin (Promega). Because all of the cysteine residues in the samples were previously alkylated, further reduction with DTT and alkylation with iodoacetamide was not performed.

MALDI-TOF MS Analysis of Tryptic Peptides—Digests (1 μl) were mixed with 1 μl of α-cyano-4-hydroxy-trans-cinnamic acid (10 mg/ml in 0.1% trifluoroacetic acid, acetonitrile, 1:1, v/v). The mixture (1 μl) was deposited onto a fast evaporation nitrocellulose matrix surface, washed twice with 2 μl of 5% formic acid, and analyzed with a TOFSpec 2E MALDI/TOF mass spectrometer (Micromass, Manchester, UK) in reflectron mode. The mass axis was adjusted using a trypsin autolysis peak (m/z 2211.10) as the lock mass. Data base search was performed to compare the monoisotopic peaks of the peptides with the theoretical molecular weights of peptides that were produced by digestion of the proteins in the Swiss-PROT data base. The maximum error allowed was set to 0.15 Da.

Molecular Mass Determination of Alkylated MT—The molecular mass of alkylated MT, separated by reversed-phase HPLC, was determined by electrospray ionization mass spectrometry (ESI-MS) using a Quattro LC mass spectrometer (Micromass, Manchester, UK). The fractions from HPLC were dried and redissolved in a solution of water/acetonitrile/trifluoroacetic acid (50:50:0.1, v/v/v), and introduced into the mass spectrometer by direct injection at a flow rate of 0.5 μl/min. MassLynx software was used to obtain the molecular masses of proteins from their original mass spectra.

ESI-MS/MS Analysis—Tandem MS/MS was performed on a Q-TOF API-US mass spectrometer (Waters, Milford, MA). The parent ion was selectively isolated and fragmented to give a series of fragment ions. The identity of the peptide and NEM and IAA modifications of cysteine were determined from the fragmentation pattern produced by the collision-induced dissociation.

Statistical Analysis—The data were expressed as mean ± S.D. Differences were tested by Student’s t test and were considered to be significant when p < 0.05.

RESULTS

A strategy of differential alkylation of cysteine residues in MT to identify disulfide bond formation in vivo was developed. A pH of 6.0 and nitrogen-flushed buffer was used for tissue homogenization immediately after removal of the heart, in the presence of NEM, to trap the sulfhydryl groups of cysteine residues that were not present as disulfide bonds. Then, after reduction of the disulfide bonds with DTT, radiolabeled IAA was used to alkylate the cysteine thiols. Because it has been
shown that NEM thiol adducts of Zn-MT are reversible upon prolonged treatment (7.5 h) with mercaptoethanol (22), we conducted control experiments using \(^{14}\text{C}\)-labeled NEM to substantiate the differential alkylation strategy used in our sample preparation. MT-TG hearts were alkylated with \(^{14}\text{C}\)NEM and the free label was removed by gel filtration. The MT-NEM adduct was then incubated with or without DTT under the conditions described under “Materials and Methods.” After removal of the DTT and unbound \(^{14}\text{C}\)NEM by gel filtration, the MT fractions were analyzed for radioactivity. The results (data not shown) indicated an \(\sim 3\%\) loss of \(^{14}\text{C}\)NEM after DTT treatment, suggesting that under the conditions used for differential alkylation in these experiments, the reversibility of NEM modification by DTT treatment was negligible.

As shown in Fig. 1, when DTT was present, a significant incorporation of \(^{14}\text{C}\) was observed in both DOX-treated and control hearts, whereas no \(^{14}\text{C}\) incorporation was observed when DTT was absent, indicating the existence of disulfide bonds. Western blotting confirmed the identity of MT in the band of the corresponding molecular mass (Fig. 1), which was the only band observed in the presence or absence of DTT. This suggests that there are no detectable dimers or multimers of MT and that the disulfide bonds were apparently formed intramolecularly, although the possibility of mixed disulfides with low molecular weight thiols such as glutathione cannot be ruled out. Quantification of the autoradiographic results by densitometric scanning revealed that DOX treatment enhanced the formation of disulfide bonds by about 33%.

To further characterize the nature of the MT disulfide bonds, the

**FIGURE 1.** Detection of intramolecular disulfide bond formation in MT of MT-transgenic mouse hearts. Protein samples were prepared as described in the sample preparation under “Materials and Methods.” The samples were then subjected to SDS-PAGE on a 15% gel in the absence of reducing agent. The gels were exposed to the autoradiography films at \(-80\, ^\circ\text{C}\) for 7 days (bottom panel). Parallel gels were electroblotted onto polyvinylidene difluoride membranes and incubated with a mouse anti-MT antibody and horseradish peroxidase-conjugated secondary antibody, followed by ECL detection (upper panel).

**FIGURE 2.** RP-HPLC separation of differentially alkylated MT molecules. A, samples prepared as described under “Materials and Methods” were run on a C4 RP-HPLC column; fractions were collected for further analysis. Lines without a marker are the absorbance at 214 nm of DOX-treated (black line) and control samples (gray line). Marked lines denote \(^{14}\text{C}\) incorporation of DOX-treated (■) and control (□) samples. B, collected fractions were electroblotted onto polyvinylidene difluoride membranes and probed by an anti-MT antibody. C, summary of radioactive fractions. Fractions from 17 to 33 and fraction 22, from the HPLC separation shown in Fig. 2A, were counted for \(^{14}\text{C}\) incorporation. Asterisk, significantly different from control (\(p < 0.005\), Student’s t test, \(n = 4\)).
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FIGURE 3. ESI-MS spectrum of fraction 28 from the HPLC separation of MT from mouse heart. Samples were treated as described under “Materials and Methods.” The fraction was analyzed by ESI-MS, and represents a spectrum of MT isolated from DOX-treated animals.

differentially alkylated MT preparations were separated by reversed-phase HPLC (RP-HPLC) on a C4 column. Chromatograms were monitored at 214 nm and showed little difference between the DOX-treated and control samples (Fig. 2A). The variation from sample to sample was minor as well (data not shown). Immunoassay of all RP-HPLC fractions revealed that both DOX-treated and control samples had peaks corresponding to MT (Fig. 2B). The first MT-immunopositive peak showed considerable 14C incorporation, whereas the second peak had negligible 14C incorporation, indicating that MT was present in forms with and without disulfide bond(s). The different retention times of the two MT-related peaks may reflect differences in the polarity of the MT molecules because of differential alkylation. Samples from DOX-treated animals showed a significant increase (p < 0.05) in 14C incorporation as shown in Fig. 2C.

The two major MT-containing fractions separated by RP-HPLC were analyzed by mass spectrometry to identify the nature of these modifications. Human MT-IIA has a metal-free molecular mass of 6042 Da. Modification of one cysteine residue by NEM will add 125.1 Da. It has been shown that MT-IIA is usually acetylated at the N-terminal, which adds an additional 42 Da. In the MT fraction containing no 14C incorporation (fraction 28), the most intense peak in the ESI-MS spectrum was at 8585 Da (Fig. 3). This agrees well with the molecular mass calculated for human MT-IIA derivatized with NEM at all 20 cysteine residues (M_r = 8604.47). To further confirm the identity of the NEM-modified MT in this fraction, mass spectral analysis was performed. Following trypsin digestion and mass analysis using MALDI-TOF/MS, seven peptide fragments were detected (Fig. 4A) and could be assigned to peptides derived from human MT-IIA with modification of all 20 cysteines by NEM. These fragments had mass errors ranging from 0.01 to 0.1 Da and covered 100% of the amino acid sequence (Table 1). This result indicates that all the cysteine residues in MT in the heart could be alkylated by NEM under the experimental conditions, and thus excluded the possibility of artificial disulfide bond formation during the sample processing conducted in this study.

HPLC fraction 22 was digested with trypsin and subjected to MALDI-TOF/MS analysis. Five individual experiments were performed and diverse mass profiles were obtained. A representative example is shown in Fig. 4B. Efficient digestion of MT by trypsin should generate eight peptides and one lysine, of which two peptides (amino acids 21–22 and 23–25) in MT and the lysine (31) are too small to be assigned precisely by MALDI-TOF/MS. However, peptide masses covering the complete sequence of MT have been observed when trypsin miscuts were allowed. Table 2 shows all of the observed masses that were matched with tryptic peptides alkylated differentially by NEM and IAA in five experiments. Most of the peptides detected were in multiply modified forms, and the same cysteine residue could be modified by NEM or IAA among different experiments or in the same experiment. For example, the two cysteines in peptide 21–25 were both modified by NEM (M_r = 860.47) or IAA (M_r = 724.23). Similarly, peptides 21–30, 32–43, and 44–51 were observed in multiple modified forms. However, the N-terminal tryptic fragment 1–20 in the β-domain was only observed with all of its five cysteine residues modified by NEM. Other modifications were not detected. Moreover, this peptide was observed both in control and DOX-treated animals. This indicates that these five cysteine residues in the N-terminal are unlikely to be involved in disulfide bond formation, suggesting some resistance to oxidation. Only one form of peptide 57–61 was detected. Nevertheless, the alternative modifications of the same cysteine residue by either NEM or IAA among different experiments or in the same experiment undoubtedly reflect the dynamic nature of disulfide bond formation between the multiple cysteine residues in MT. The apparent rapid exchange among metal-bound and disulfide bonded cysteines precludes repeatedly capturing a single species. On the other hand, the most abundant peptide observed in all five experiments performed on control and DOX-treated hearts, was peptide 14 with a mass of 1064.49 Da, in which two of the three cysteine residues were involved in disulfide bond formation. Tandem MS/MS spectrometry allowed us to identify the specific cysteines involved in disulfide bond formation. The doubly charged ion of peptide 14, at m/z 533.3, was selected for sequencing. The product ion spectrum for this peptide is illustrated in Fig. 4C. Fragments b1, b2, and b3, which contain cysteine 44, are 57.0 Da larger than the calculated masses without alkylation, indicating that cysteine 44 was IAA-modified. Similarly, analysis of the y2 ion, which contains cysteine 50, shows it is 125.1 Da more than calculated without alkylation, indicating that cysteine 50 was alkylated by NEM. Further analyses of y4, y5, y6, and y7 ions, which contain cysteine 48 and cysteine 50, showed that they were 182.1 Da more than calculated without cysteine alkylation. Considering that cysteine 50 was shown above to be alkylated by NEM, thus cysteine 48 must be modified by IAA. Therefore, cysteine 44 and cysteine 48 were involved in disulfide bond formation.

DISCUSSION

One of the unique properties of MT is its peculiar amino acid composition, characterized by the absence of aromatic amino acids or histidine and the high cysteine content (~30% of the total amino acid residues). The distribution of cysteine in the amino acid sequence allows the formation of tetrathiolate clusters that confer on MT a highly stable tertiary structure capable of binding heavy metals with high affinity. Although the sulfhydryl residues of cysteine become firmly complexed upon metal binding, studies have shown that the thiol groups remain reactive under oxidative or nitrosative stress conditions (16, 20). In addition, when MT is exposed to an excess of dithiodipyridine, all 20 of its cysteines are oxidized within 1 h with the concomitant release of all 7 zinc atoms (23). Also, reaction of MT with 5,5′-dithiobis-2-nitrobenzoic acid caused the formation of both intra- and intermolecular Cys–Cys disulfides and a small number of mixed disulfides (17). MT dimers, formed via disulfide bond formation between cysteines in the α-domain, have also been identified by mass spectrometry (24). However, none of these findings have been demonstrated in vivo.

Attempts to demonstrate the formation of MT disulfide bonds in vivo...
FIGURE 4. Mass spectra of tryptic fragments of the HPLC fractions 22 and 28 in Fig. 2. Representative MALDI-TOF/MS spectra of HPLC fractions 28 (A) and 22 (B) in Fig. 2. An asterisk denotes the peptides that are illustrated in Tables 1 and 2. C, tandem MS/MS spectrum of the doubly charged ion at m/z 533.3 derived from peptide 14 in Table 2.

TABLE 1
Masses of MT-IIA tryptic peptides observed in RP-HPLC fraction 28 (Fig. 2)

| Peptide* | Positionb | Domain | Calculatedc (Da) | Errord |
|----------|------------|--------|------------------|--------|
| 1 *MDPNCSCAAGDSCTCAGSCK | 1–20 | β | 2591.17 | 0.05 |
| 2 CKECK | 21–25 | β | 860.47 | 0.05 |
| 3 CKECKCTSCCK | 21–30 | β | 1632.86 | 0.06 |
| 4 CTSCCK | 26–31 | α | 919.51 | 0.1 |
| 5 SCCSCCPVGCAK | 32–43 | α | 1785.90 | 0.07 |
| 6 CAQGCIICK | 44–51 | α | 1200.64 | 0.06 |
| 7 GASDKCSCCA | 52–61 | α | 1319.63 | 0.01 |

* Tryptic digestion fragments from MT-IIA.

b Amino acid sequence position.

c Masses are the calculated monoisotopic masses for the sequences in the table.

d Errors are the difference between the calculated monoisotope masses and the masses detected in the samples. Bold letters in the sequence are the cysteine residues alkylated by NEM. The asterisk indicates that the amino acid M is acetylated.
was impracticable in the past largely because of the fact that the tissue concentrations of MT were insufficient. Use of MT-TG mice, in which MT is overexpressed specifically in the heart, allowed us to overcome this limitation. Previous experiments have shown that the cadmium-binding capacity of MT in the MT-TG mouse heart under DOX treatment was reduced compared with saline-treated controls (25). This observation suggested that MT disulfide bond formation could be responsible for the decrease in metal binding capacity. In the present study, NEM was used to trap the metal-bound and any free sulfhydryl groups. The methodology used effectively prevented artifactual disulfide bond formation during tissue processing. Following reduction by DTT, the IAA-modified cysteine residues thus represented the MT disulfides in vivo. The concomitant release of zinc accompanying disulfide bond formation would be an important component of the involvement of MT in zinc homeostasis. Under oxidative stress conditions, increased disulfide bond formation in MT was observed, suggesting that additional zinc release occurs in response to oxidative stress.

A number of *in vitro* studies have shown that MT can form dimers through disulfide bond formation under oxidative conditions (17, 19, 24), although intramolecular oxidation of the cysteine thiolates and mixed disulfide formation has also been demonstrated (28). Intramolecular disulfide bonds following treatment of MT with 5-nitrosocysteine, which generates nitric oxide, were also detected by Raman spectroscopy (16). Structurally, the 20 cysteines of MT are sequentially very close, which would seemingly favor intramolecular disulfide bond formation (10).

The factors that control the partitioning between inter- and intramolecular disulfide bond formation under *in vitro* and *in vivo* conditions are largely unknown. One possibility is that the MT concentration *in vivo* is significantly lower than that *in vitro*, thus favoring intramolecular disulfide bond formation *in vivo*. It should be noted that the resolution of the SDS-PAGE, used in our present study, precludes the detection of small molecular weight differences. Therefore, the possibility of mixed disulfide bond formation between MT and low molecular weight thiols, such as glutathione, cannot be ruled out. However, if a thiol group in a MT to NO led to the selective release of three metals from the MT-domain, whereas leaving the four metals in the α-domain unchanged (11). The concomitant formation of disulfide bonds in the β-domain was demonstrated using $^{1}$H and $^{13}$C NMR spectroscopy (11). However, the use of Cd-$\alpha$-MT in these experiments may modify the cysteine reactivity, because Cd-MT is considered to be more stable than Zn-MT (10, 29, 30). The function of MT in the detoxification of heavy metals has been attributed to the tight metal binding in the α-domain, whereas the

### Table 2

| Peptide | Position | Domain | Calculated | Error | Number of Cys modified by NEM | IAA | Experiment | 1 | 2 | 3 | 4 | 5 |
|---------|----------|--------|------------|-------|-----------------------------|-----|------------|---|---|---|---|---|
| 1 MDPNCSGAAGDSCTCAGSCK (α) | 1–20 α | 2591.17 | 0.15 | 5 | 0 | ___ | ___ | ___ | ___ | ___ | ___ | ___ |
| 2 CKECK | 21–25 β | 723.31 | 0.09 | 0 | 2 | ___ | ___ | ___ | ___ | ___ | ___ | ___ |
| 3 CKECK | 21–25 β | 867.13 | 0.15 | 0 | 2 | ___ | ___ | ___ | ___ | ___ | ___ | ___ |
| 6 CKECK | 21–30 β | 1360.55 | 0.14 | 0 | 2 | ___ | ___ | ___ | ___ | ___ | ___ | ___ |
| 7 CKECK | 23–30 β | 1276.66 | 0.07 | 0 | 2 | ___ | ___ | ___ | ___ | ___ | ___ | ___ |
| 8 CTSCK | 26–30 β | 791.41 | 0.10 | 0 | 2 | ___ | ___ | ___ | ___ | ___ | ___ | ___ |
| 9 CTSCK | 26–31 β | 919.51 | 0.07 | 0 | 2 | ___ | ___ | ___ | ___ | ___ | ___ | ___ |
| 10 SCCSCCPVCGAK | 32–43 α | 1445.51 | 0.08 | 0 | 2 | ___ | ___ | ___ | ___ | ___ | ___ | ___ |
| 11 SCCSCCPVCGAK | 32–43 α | 1649.75 | 0.13 | 3 | 2 | ___ | ___ | ___ | ___ | ___ | ___ | ___ |
| 12 SCCSCCPVCGAK | 32–56 α | 3221.51 | 0.17 | 5 | 3 | ___ | ___ | ___ | ___ | ___ | ___ | ___ |
| 13 CAQGCICK | 44–51 α | 996.51 | 0.12 | 0 | 3 | ___ | ___ | ___ | ___ | ___ | ___ | ___ |
| 14 CAQGCICK | 44–51 α | 1064.49 | 0.07 | 1 | 2 | ___ | ___ | ___ | ___ | ___ | ___ | ___ |
| 15 CAQGCICK | 44–51 α | 1132.56 | 0.11 | 2 | 1 | ___ | ___ | ___ | ___ | ___ | ___ | ___ |
| 16 CSCCA | 57–61 α | 793.34 | 0.07 | 2 | 1 | ___ | ___ | ___ | ___ | ___ | ___ | ___ |

* a: Tryptic digestion fragments from MT-IIA.
* b: Amino acid sequence position.
* c: Masses are the calculated monoisotopic masses for the sequences in the table.
* d: Errors are the largest difference between the calculated monoisotope masses and the masses detected in the samples in 5 experiments. Bold letters in the sequence are the cysteine residues alkylated by either NEM or IAA. The asterisk indicates that the amino acid M is acetylated.
* e: Indicate that the peptides are detected in control.
* f: Indicate that the peptides are detected in DOX-treated samples, respectively.
β-domain of MT has been proposed to be important for zinc and copper homeostasis (31). However, an intermolecular disulfide bond in the α-domain of MT has been demonstrated in vitro under oxidative conditions (19). Our results suggest that both the α- and β-domains of MT may be involved in disulfide bond formation under both physiological and oxidative stress conditions. It has been concluded that the lability of each cluster toward various reagents is likely to be controlled by the specific steric and electronic factors that will vary from reagent to reagent (16). However, the first five cysteines in the N-terminal β-domain are likely resistant to oxidation. In addition, tandem MS/MS spectrometry allowed us to determine that cysteine 44 and cysteine 48 were often involved in disulfide bond formation. This observation does not support the notion derived from in vitro studies, that only the β-domain of MT is important for homeostasis of zinc (31). Instead, our results suggest that the α-domain of MT is likely involved in zinc homeostasis under physiological conditions. It should be pointed out that the oxidant(s) generated by DOX treatment may be different from that by NO in terms of specificity with regard to the specific cysteine oxidized. However, this study provides additional support for an antioxidant role for MT in protecting heart tissue from oxidative damage that accompanies the use of DOX in cancer chemotherapy (1, 3, 25).

In summary, the data obtained from the present study demonstrate that MT disulfide bonds exist in vivo under both physiological and oxidative stress conditions. The MT disulfide bonds are likely to be intramolecular and both α- and β-domains of MT are involved in disulfide bond formation. The α-domain appears to be more easily oxidized than the β-domain in mouse hearts. Under physiological conditions, the formation of MT disulfide bonds could be involved in the regulation of zinc homeostasis via zinc release from MT. Additional zinc release from MT under oxidative stress conditions would be accompanied by increased MT disulfide bond formation.

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