J. Biochem. 132, 217–221 (2002)

Interactions of Arsenic with Human Metallothionein-2[1]

Mitsutoshi Toyama,[*] Mitsuo Yamashita,[*] Noriaki Hirayama,[†] and Yoshikatsu Murooka[*,2]

[*]*Department of Biotechnology, Graduate School of Engineering, Osaka University, Yamada-oka, Suita, Osaka 565-0871; and* [†]*Department of Biological Science and Technology, Tokai University, Nishino, Numazu, Shizuoka 410-0321*

Received February 12, 2002; accepted May 20, 2002

Arsenic is a toxic element that is found in the atmosphere, as well as in aquatic and terrestrial environments. We have demonstrated that As^{3+} binds to human metallothionein-2 (hMT-2) by UV absorption spectroscopy, inductively coupled plasma–atomic emission spectrometry (ICP-AES), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). MALDI-TOF-MS revealed that the structure of the adduct formed by arsenic and hMT-2 (As-hMT-2) was not homogeneous. The maximum molar ratio of arsenic to hMT-2 was found to be more than 6:1 on ICP-AES, UV absorption spectroscopy and MALDI-TOF-MS. The ratio of the number of sulfhydryl groups in hMT-2 that bound arsenic was 3:1, which is the same as the ratios reported previously for arsenic-glutathione and arsenic-phytochelatin complexes.

Key words: arsenic, heavy metal binding protein, human metallothionein, MALDI-TOF-MS.

Arsenic is a toxic element that is found ubiquitously in the environment. Substantial amounts of arsenic are released as a result of geological activity, and as a result of the disposal of industrial waste products and the combustion of coal, which accounts for the emission of 1.2×10^4 to 2.6×10^4 tons of arsenic into the atmosphere per year (*1*). Arsenic binds to sulfhydryl groups (*2*), and arsenic intoxication can be treated by the administration of dithiols, such as 2,3-dimercaptopropanol or 1,2-ethanedithiol (*3, 4*), which compete with proteins for ingested arsenic.

Metallothioneins (MTs) are relatively small (~7 kDa), cysteine- and metal-rich proteins. MT was first isolated from equine renal cortex 40 years ago (*5*), and equine MT contains 61 amino acids, of which 20 are cysteine residues. Other MTs were subsequently isolated from the kidneys, livers, and intestines of a variety of animal species, as well as from fungi, plants (*6*), and metal-resistant bacteria (*7, 8*). The two major isoforms of mammalian MT (MT-1 and MT-2) have similar not identical amino acid sequences, and a slight difference in overall charge. The characterization of a recently discovered growth-inhibitory factor in human brain tissue and nerves as a metallothionein (MT-3) has stimulated new interest in the study of this family of small proteins (*9, 10*).

The functions of MT are still not fully understood. The protein appears to play a fundamental role in the metabolism of copper and zinc ions under various physiological conditions (*11, 12*), and is able to donate metal ions to apo-Zn^{2+} enzymes (*13, 14*). MT might also be important for the sequestration of toxic Cd^{2+} ions, thereby preventing reactions with other cellular targets in mammals and other higher organisms (*15*). MT also appears to play a role in the scavenging of free radicals, in response to stress, and in the pharmacology of alkylating agents (*16*).

Arsenic induces the expression of the gene for MT in rat liver *in vivo* (*17*), and exposure to zinc, an effective inducer of the synthesis of MT, prevents arsenic toxicity in some instances (*18*). By contrast, activation of the gene for MT prior to exposure of rat myoblast (L6) cells to As^{3+} *in vitro* appears to have little impact on the overt cytotoxicity or molecular toxicity of the metalloid ion (*19*). The role of MT in preventing the toxic effects of many inorganic compounds has been clarified, but the detoxification of arsenic by MT, *via* the binding of arsenic to MT, has not been confirmed.

We recently succeeded in inducing high-level expression of the human gene for MT-2 (h*MT-2*) in *Escherichia coli* and developed a simple system for the purification of MT-2 (*20*). Our system not only provides a straightforward method for purification of the fusion protein but also allows purification of the native peptide without any additional amino acids. We report here the reaction of As^{3+} with human MT, as studied by UV absorption spectroscopy, inductively coupled plasma-atomic emission spectrometry (ICP-AES), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). This is the first report, to our knowledge, of the isolation, stoichiometry and structures of a complex between As^{3+} and hMT-2.

MATERIALS AND METHODS

Expression of hMT-2—Plasmid pTIMT1, constructed previously and including the gene for intein-fused hMT-2 (*20*),

[1]This work was supported by a Grant-in-Aid (No. 11450315) from the Ministry of Education, Science, Sports and Culture of Japan.
[2]To whom correspondence should be addressed. Phone: +81-6-6879-7416, Fax: +81-6-6879-7418, E-mail: murooka@bio.eng.osaka-u.ac.jp
Abbreviations: hMT-2, human metallothionein-2; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; As-hMT-2, complex of arsenic and hMT-2; ICP-AES, inductively coupled plasma–atomic emission spectrometry; As+Apo-hMT-2, mixture of arsenic and Apo-hMT-2.

was used to generate hMT-2 in *E. coli* ER2566 (New England Biolabs, Beverly, MA). Cells harboring pTIMT1 were cultured overnight at 37°C in 100 ml of LB broth (Difco Laboratories, Detroit, MI) containing 100 μg/ml ampicillin (Ap). A suspension of cultured cells (4 ml) was added to 400 ml of super broth medium [1.1% (w/v) tryptone, 2.2% (w/v) yeast extract, 0.45% (w/v) glycerol, 1.3% (w/v) K_2HPO_4, 0.38% (w/v) KH_2PO_4], supplemented with 100 μg/ml Ap, in a two-liter flask. The culture was incubated, with shaking, at 37°C until the mid-logarithmic phase of growth. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and then the incubation was continued overnight at 20°C.

Purification of hMT-2—Cells were harvested from the culture broth by centrifugation (8,000 ×*g*, 5 min, 4°C), suspended in 200 ml of column buffer (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM EDTA), and then disrupted by sonication on ice. The sonicate was centrifuged (8,000 ×*g*, 10 min, 4°C) and the supernatant was collected. A suspension of chitin resin (8 ml; New England Biolabs) was added to the supernatant and then the mixture was incubated overnight at 4°C. Chitin-bound intein-fused hMT-2 was collected by centrifugation (2,500 ×*g*, 20 min, 4°C). The pellet was washed three times with 30 ml of column buffer. After the addition of DTT to a final concentration of 100 mM and incubation for 20 h at 20°C, the mixture was poured into a disposable chromatography column (Econo-Pac; Bio-Rad, Hercules, CA) and the resin was washed with 5 ml of the column buffer. The eluate, which contained hMT-2, was immediately acidified to pH 1 with 12 N HCl and then centrifuged at 10,000 ×*g* for 1 min to remove any solid materials. The clear supernatant was loaded onto a column (1 cm i.d. × 120 cm) of Sephadex G-50 (Amersham Pharmacia Biotech AB, Uppsala, Sweden), which had been equilibrated with degassed 0.012 N HCl, and eluted with the same solution. The fractions containing Apo-hMT-2, namely, the metal-free form of MT, were identified by monitoring the absorption at 220 nm (A_{220}) and collected.

Reconstitution of hMT-2—A 10-fold molar excess of arsenic trioxide was added to a solution of purified Apo-hMT-2 that had been saturated with nitrogen gas, and the pH of the mixture was adjusted to 8.6 with 2 M Tris base. The sample was loaded onto a column (1 cm i.d. × 120 cm) of Sephadex G-50, which had been equilibrated with degassed 20 mM Tris-HCl, pH 7.4, and then the column was eluted with the same solution to separate hMT-2 from excess cadmium or arsenic ions. Fractions that contained the complex of arsenic with hMT-2 (As-hMT-2) were collected by monitoring A_{220} and A_{250}.

UV Absorption Spectroscopy of As-MT—A solution of 0 to 560 μM arsenic trioxide (50 μl) and a solution of 200 mM Tris-HCl (pH 7.8) (50 μl) were added to a 28 μM solution of purified Apo-hMT-2 (100 μl), it being confirmed that the pH of the resultant solution was 7.4. Then we recorded absorption spectra, from 220 to 320 nm, of 140 μM arsenic trioxide in 50 mM Tris-HCl at pH 7.4, of Apo-hMT-2 at pH 2, and of a mixture of arsenic trioxide and Apo-hMT-2 (As+Apo-hMT-2) at pH 2 and 7.4 with a spectrophotometer (model U-3000; Hitachi, Tokyo).

Quantitation of Protein in Apo-hMT-2—To determine the concentration of Apo-hMT-2, we measured the level of sulfhydryl groups in the purified protein using 2,2′-dithiodipyridine (ε_{343} = 7,600 M^{-1} cm^{-1}; *21*).

SDS-PAGE—Proteins at each step of the purification were separated by SDS-PAGE as described by Laemmli (*22*). Proteins were detected by silver staining with a Silver Stain II Kit Wako (Wako Pure Chemicals, Osaka).

Determination of Arsenic Concentrations—The concentrations of arsenic were determined by ICP-AES (model ICP-2000; Shimadzu, Kyoto).

MALDI-TOF-MS—Arsenic trioxide was added at 5 mM to a solution of purified Apo-hMT-2 (14 μM) and then the mixture was incubated 30 min at 25°C. Then 1 μl of the mixture was added to 30 μl of the matrix solution [10 mg/ml sinapic acid, 0.1% (v/v) trifluoroacetic acid, 50% (v/v) acetonitrile] for analysis by mass spectrometry. Two microliters of the sample-matrix mixture was put on the sample plate and the solvent was removed by evaporation. The molecular weight of As-hMT-2 was determined with a MALDI-TOF mass spectrometer (Voyager™ RP; Applied Biosystems, Foster, CA). The instrument was calibrated with insulin (mol. wt. 5734.59; Sigma, St. Louis, MO) and ubiquitin (mol. wt. 8565.89; Sigma) as standard proteins.

RESULTS

Purification of Apo-hMT-2 and As-hMT-2—A fusion protein consisting of intein and hMT-2 was produced in *E. coli*. The fusion protein was purified with chitin resin and cleaved with DTT. Then hMT-2 was purified to homogeneity (Fig. 1). The apparent molecular mass of hMT-2 was estimated to be 15 to 16 kDa by SDS-PAGE, this value being much higher than the calculated molecular mass of 6 kDa. However, the phenomena that MT shows a broad band and a high apparent molecular weight on SDS-PAGE are in agreement with previous reports (*23–26*).

UV Absorption Spectroscopy of As-MT—We analyzed purified Apo-hMT-2 and As+Apo-hMT-2 at the same protein concentrations by UV absorption spectroscopy at pH 2 and pH 7.4 (Fig. 2A). The absorbance of As+Apo-hMT-2 increased between 220 and 320 nm at pH 7.4 [Fig. 2A(a)], whereas no absorbance of Apo-hMT-2 was observed between 260 and 320 [Fig. 2A(b)]. These UV absorption spectra were similar to the absorption spectrum of a complex

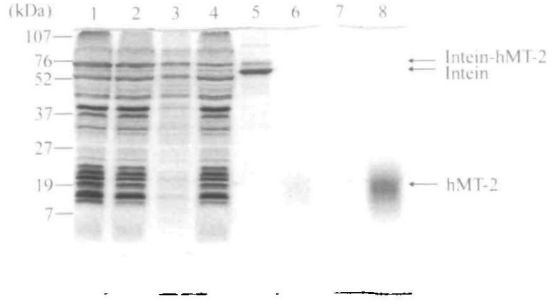

Fig. 1. **Expression of the intein-hMT-2 fusion protein and purification of Apo-hMT-2.** The results are shown for the cell extract after cell disruption (lane 1), the soluble fraction of the cell extract (lane 2), the insoluble fraction of the cell extract (lane 3), the supernatant with added chitin resin (lane 4), the remaining protein eluted from the chitin resin with SDS after elution with DTT (lane 5), the fraction eluted from the chitin resin with DTT (lane 6), and the Apo-hMT-2 fraction after gel filtration (lanes 7 and 8). Protein samples were separated by SDS-PAGE (15% polyacrylamide). Lanes 1 to 7 were loaded with proteins equivalent to 80 μl of culture, and lane 8 was loaded with 1 μg of hMT-2.

between arsenic and rabbit MT (As-rMT) (*27*). A small increase in absorbance of As+Apo-hMT-2 [Fig. 2A(c)] as compared with Apo-hMT-2 at pH 2 [Fig. 2A(d)] was also observed. These results suggest that arsenic interacts with hMT-2 at neutral pH, but very weakly at acidic pH. To determine the saturation concentration of arsenic binding hMT-2, we measured UV absorption spectra at 220, 240, and 260 nm, respectively, of various concentrations of arsenic (0 to 280 μM) with 14 μM Apo-hMT-2 (Fig. 2B). The absorption in the spectrum of As-hMT-2 at 220 nm (pH 7.4) greatly increased to the molar ratio of arsenic to hMT-2 of six and very slowly increased with increasing molar ratio of arsenic to hMT-2 of more than six. The molar extinction coefficients of As-hMT-2 containing 280 μM arsenic were calculated to be 12.9×10^4, 5.0×10^4, 2.5×10^4, 1.5×10^4, and 0.8×10^4 M^{-1} cm^{-1} at 220, 240, 260, 280, and 300 nm, respectively. The absorbance of As-hMT-2 at pH 2 increased very slowly, corresponding with the increasing molar ratio of arsenic to hMT-2. From these results, we used the molar ratio of arsenic to hMT-2 of 20 for further studies for preparation of As-hMT-2.

Concentration of Protein in As-hMT-2—When arsenic and hMT-2 were present together in solution, we were unable to quantitate sulfhydryl groups using 2,2′-dithiodipyridine (data not shown). Therefore, we tried to measure the concentration of protein in As-hMT-2 by monitoring UV absorption. We measured the changes in UV absorbance of As+Apo-hMT-2 and Apo-hMT-2 during incubation at 4°C for 12 h. The changes in the absorption of UV light by Apo-hMT-2 (pH 2 and pH 7.4) and As+Apo-hMT-2 (pH 7.4) from 30 min to 12 h after the start of incubation are shown in Fig. 3. At pH 2, the UV absorption spectrum of Apo-hMT-2 remained unchanged during the incubation. At pH 7.4, the absorption of UV light by Apo-hMT-2 and As+Apo-hMT-2 changed in the range from 220 to 260 nm. These results indicate that the molar extinction coefficient of As-hMT-2 from 220 to 280 nm differed before and after gel filtration. Since the absorption at 280 nm was unchanged during overnight storage, we used the molar extinction coefficient at 280 nm to measure the concentration of hMT-2 in As-hMT-2.

Stoichiometry of the Binding of Arsenic to hMT-2—To determine the stoichiometry of the binding of arsenic to hMT-2, we mixed hMT-2 with arsenic trioxide and then measured the concentration of arsenic in purified As-hMT-2 by ICP-AES. The concentrations of arsenic and protein in purified As-hMT-2 were determined to be 36 ± 1 and 6.7 ± 0.7 μM, respectively. The molar ratio of arsenic to hMT-2 was calculated to be 5.4 ± 0.8:1.0. The molar ratio of arsenic to hMT-2 was lower than the molar ratios of other heavy metals to hMT-2. The ratios for Cd, Zn, Hg, and Cu have been reported to be more than 7:1 (*20, 28–30*).

We performed MALDI-TOF-MS to determine the molar ratio of hMT-2 to arsenic at the molecular level. Since As-hMT-2 showed the spectral peak only at low pH with 0.1% TFA, we used 10 mM arsenic trioxide and 14 μM hMT-2 protein. The spectrum of As-hMT-2 had seven sharp peaks (Fig. 4). The molecular weight of the material in each of the

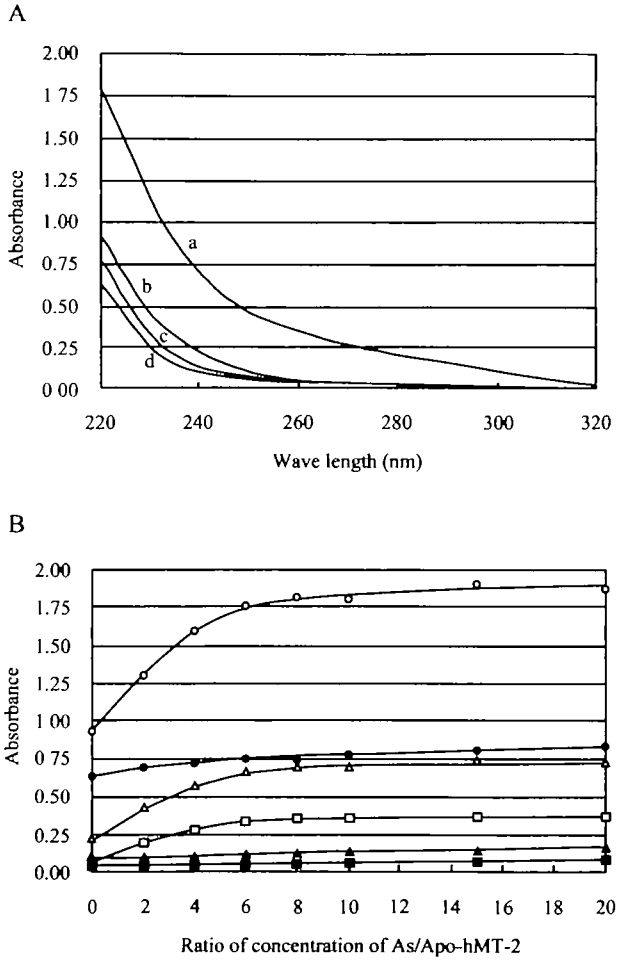

Fig. 2. **Absorption spectra of Apo-hMT-2 and As+Apo-hMT-2.** A: Absorption spectra were recorded using solutions containing 14 μM hMT-2 with or without or 280 μM arsenic at 25°C. As+Apo-hMT-2 in 50 mM Tris-HCl, pH 7.4 (a); Apo-hMT-2 in 50 mM Tris-HCl, pH 7.4 (b); As+Apo-hMT-2 in 10 mM HCl, pH 2 (c); and Apo-hMT-2 in 10 mM HCl, pH 2 (d). B: UV absorption spectra were recorded using solutions containing 14 μM hMT-2 and various concentrations of arsenic (0 to 280 μM) at 25°C. As+Apo-hMT-2 in 50 mM Tris-HCl, pH 7.4 at 220 nm (open circles), 240 nm (open triangles), and 260 nm (open squares), respectively; As+hMT-2 in 10 mM HCl, pH 2, at 220 nm (closed circles), 240 nm (closed triangles), and 260 nm (closed squares), respectively.

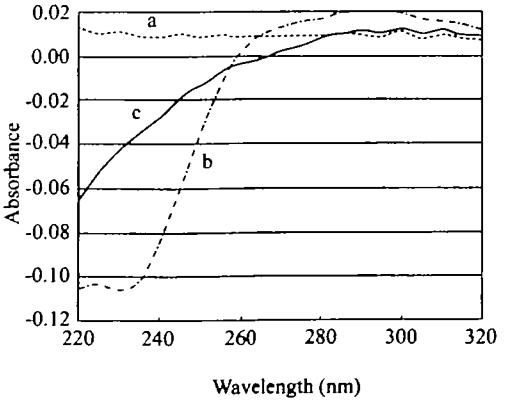

Fig. 3. **Changes in the absorption of Apo-hMT-2 and As-hMT-2 from 30 min to 12 h after the start of incubation.** Apo-hMT-2 in 10 mM HCl, pH 2 (a); Apo-hMT-2 in 40 mM Tris-HCl, pH 7.4 (b); and As+Apo-hMT-2 in 40 mM Tris-HCl, pH 7.4 (c).

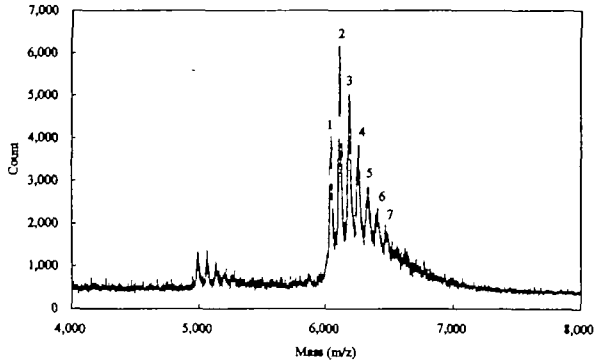

Fig. 4. **Molecular weights of As-hMT-2 complexes, as determined by MALDI-TOF-MS.** A mass spectrum was recorded for a solution of As-hMT-2 (0.9 pmol) in 50% acetonitrile that contained 10 mg/ml sinapic acid and 0.1% trifluoroacetic acid. Insulin and ubiquitin were used as molecular weight standards for molecular weight determinations. The analysis revealed the characteristic signal of Apo-hMT-2 (peak 1: m+H$^+$ 6,043) and presumed As-hMT-2 (peaks 2–7: m+H$^+$ 6,114, 6,187, 6,257, 6,329, 6,403, 6,476). The signal at peaks 2 to 7 corresponds to the protonated complex formed by hMT-2 binding 1 to 6 arsenic molecules (atomic weight: 74.9) *via* each of three thiolate coordinations (deprotonated sulfhydryl groups).

TABLE I. **Molecular weights of As-hMT-2 complexes.**

Peak no.	Molecular weight[a]	Difference from molecular weight of peak 1	ΔM_n[b]
1	6,043 ± 1	0	0.0
2	6,114 ± 1	71 ± 2	71.9
3	6,187 ± 1	144 ± 2	143.8
4	6,257 ± 1	214 ± 2	215.7
5	6,329 ± 1	286 ± 2	287.6
6	6,403 ± 1	360 ± 2	359.5
7	6,476 ± 1	433 ± 2	431.4

[a]As measured by MALDI-TOF-MS.
[b]$\Delta M_n = (A - 3B)(n - 1)$; where A is the atomic weight of arsenic (74.9), B is the atomic weight of hydrogen (1.0), and n is the peak number in Fig. 4.

seven peaks was determined to be 6,043, 6,114, 6,187, 6,257, 6,329, 6,403, and 6,476, respectively, with insulin and ubiquitin as the standards. Moreover, some small peaks were observed on the higher molecular weight side of peak 7. The theoretical molecular weights of Apo-hMT-2 and its protonated form are 6,042 and 6,043, respectively, which correspond to the molecular weight of the material in peak 1 in Fig. 4. These data suggest that peak 1 in Fig. 4 represents Apo-hMT-2. The values after subtraction of the molecular weight of the material in peak 1 from those of the materials in peaks 2 through 7 are 71, 144, 214, 286, 360, and 433 (Fig. 4 and Table I). The presence of peaks 2 through 7 suggests that As-hMT-2 complexes with multiple structures exist together with Apo-hMT-2. The atomic weights of arsenic and hydrogen are 74.9 and 1.0, respectively, and the molecular weights values, ΔM_n, for peaks 1 to 7 minus the molecular weight for peak 1 conform to the following equation:

$$\Delta M_n = (A - 3B)(n - 1),$$

where A is the atomic weight of arsenic, B is the atomic weight of hydrogen, and n is the peak number (n = 1 to 7; Table I). This suggests that the binding of one arsenic ion resulted in the loss of three protons from hMT-2.

DISCUSSION

Metallothioneins appear to play important roles in human health and disease, as well as in the mechanisms of action of certain therapeutic agents, as, for example, in the case of cellular resistance to platinum-containing anti-cancer drugs (*31, 32*). The synthesis of MTs is induced in biological systems by metal ions, and the synthesis of MTs may provide protection from toxicity *via* the sequestration of such metal ions (*33*). Arsenic can enhance the expression of the *MT* gene, at least *in vivo* (*17*). The direct toxic effect of arsenic is thought to be the result of its interaction with sulfhydryl groups in proteins (*34, 35*). The direct binding of arsenic to proteins seems to inhibit their functions.

When a metal binds to a sulfhydryl group in a protein, a change in absorption may occur in the wavelength range from UV to visible. The absorption spectrum of As-rMT has a shoulder (*27*). In this study, we noted a slight shoulder in the absorption spectrum of As-hMT-2. We tried to measure the concentrations of protein in Cd-hMT-2 and Apo-hMT-2 by measuring the concentrations of sulfhydryl groups. However, in the case of As-hMT-2, we failed to detect sulfhydryl groups with the method of Pedersen and Jacobsen (*21*) using 2,2′-dithiodipyridine. This failure might have been due to the possibility that the binding affinity of arsenic for sulfhydryl groups in hMT-2 was stronger than that of 2,2′-dithiodipyridine for these SH groups. Alternatively, the sulfhydryl groups in As-hMT-2 might have been oxidized.

The stoichiometry of the binding of metal ions to MT has been reported to vary from 7 to 10, 12, and even 20 metal ions per protein molecule, with different proposed geometries, such as digonal, trigonal, and tetrahedral (*36*). These results were obtained with monovalent metal ions, such as Au^+ (*37*) and Cu^+ (*29*), divalent metal ions, such as Zn^{2+}, Cd^{2+}, and Hg^{2+}; and trivalent metal ions, such as In^{3+}, Sb^{3+} (*29*), and Bi^{3+} (*38*). In the present study we determined the stoichiometry of the binding of As^{3+} to Apo-hMT-2 to be 5.4 ± 0.8:1.0 by ICP-AES and UV absorption spectroscopy. To our surprise, the results of MALDI-TOF-MS indicated that the structure of the As-hMT-2 complex was not homogeneous, and that the maximum molar ratio of As^{3+} to Apo-hMT-2 in As-hMT-2 was more than 6:1. These results suggest that most hMT-2 binds a full complement of arsenic at pH 7.4, but not at pH 2.

Structural analysis of As-GSH, generated through incubation of the tripeptide with arsenic, by NMR spectroscopy revealed the coordination of As^{3+} by three peptide molecules (*39*). Electrospray ionization mass spectroscopy of arsenic-phytochelatin (As-PC) revealed the coordination of As^{3+} by three cysteine residues from two molecules of PC (*40*). It is conceivable that Apo-hMT-2 coordinates arsenic similarly. The formation of the As-hMT-2 complex and the corresponding mass signal identified on MALDI-TOF-MS were in perfect accord with a structural model in which three sulfhydryl groups are provided by hMT-2 molecules for the binding of As^{3+}. Eighteen of twenty cysteine residues in hMT-2 should bind six atoms of arsenic at high affinity, judging from the linear relationship of arsenic with hMT-2 in the UV absorption spectrum analysis. A slight increase

in absorption was observed at a molar ratio of arsenic to hMT-2 of more than 6. This suggests that over seven atoms of arsenic bind weakly to the bridging cysteine or other residue in hMT-2 molecules. Thus, the arsenic-binding molecules with over seven atoms were observed as small peaks on MALDI-TOF-MS.

In this study, we succeeded in isolating an As-hMT-2 complex *in vitro*, and clearly showed that hMT-2 has arsenic-binding ability and that the structure of the complex is similar to that of complexes formed by other thiol reagents. These findings will probably facilitate the use of metallothionein as a biomaterial or bioremediation for arsenic polluting the environment.

REFERENCES

1. Nriagu, J.O. and Pacyna, J.M. (1988) Quantitative assessment of worldwide contamination of air, water and soils by trace metals. *Nature* **333**, 134–139
2. Kalef, E. and Gitler, C. (1994) Purification of vicinal dithiol-containing proteins by arsenical-based affinity chromatography in *Methods in Enzymology* (Lester, P., ed.) Vol. 233, pp. 395–403, Academic Press, New York
3. Webb, E.C. and van Heyningen, R. (1947) The action of British anti-lewisite (BAL) on enzyme systems. *Biochem. J.* **41**, 74–78
4. Whittaker, V.P. (1947) An experimental investigation of the 'ring hypothesis' of arsenical toxicity. *Biochem. J.* **41**, 56–62
5. Margoshes, M. and Vallee, B.L. (1957) A cadmium protein from equine kidney cortex. *J. Am. Chem. Soc.* **79**, 4813–4814
6. Binz, P.-A. and Kägi, J.H.R. (1999) Metallothionein: Molecular evolution and classification in *Metallothionein IV* (Klaassen, C. D., ed.) pp. 7–13, Birkhäuser Verlag, Basel, Boston, Berlin
7. Higham, D.P., Sadler, P.J., and Scawen, M.D. (1984) Cadmium-resistant *Pseudomonas putida* synthesize novel cadmium proteins. *Science* **225**, 1043–1046
8. Daniels, M.J., Turner-Cavet, J.S., Selkirk, R., Sun, H., Parkinson, J.A., Sadler, P.J., and Robinson, N.J. (1998) Coordination of Zn^{2+} (and Cd^{2+}) by prokaryotic metallothionein. Involvement of his-imidazole. *J. Biol. Chem.* **273**, 22957–22961
9. Uchida, Y., Takio, K., Ihara, Y., and Tomonaga, M. (1991) The growth inhibitory factor that is deficient in the Alzheimer's disease brain is a 68 amino acid metallothionein-like protein. *Neuron* **7**, 337–347
10. Uchida, Y. (1993) Growth inhibitory factor in brain in *Metallothionein III, Biological Roles and Medical Implications* (Suzuki, K.T., Imura, N., and Kimura, M., eds.) pp. 315–328, Birkhäuser Verlag, Basel, Switzerland
11. Richards, M.P. (1989) Recent developments in trace element metabolism and function: role of metallothionein in copper and zinc metabolism. *J. Nutr.* **119**, 1062–1070
12. Karin, M. (1985) Metallothioneins: proteins in search of function. *Cell* **41**, 9–10
13. Li, T.Y., Kraker, A.J., Frank Shaw III, C., and Petering, D.H. (1980) Ligand substitution reactions of metallothioneins with EDTA and apo-carbonic anhydrase. *Proc. Natl. Acad. Sci. USA* **77**, 6334–6338
14. Jiang, L., Maret, W., and Vallee, B.L. (1998) The glutathione redox couple modulates zinc transfer from metallothionein to zinc-depleted sorbitol dehydrogenase. *Proc. Natl. Acad. Sci. USA* **95**, 3483–3488
15. Hamer, D. (1986) Metallothionein. *Annu. Rev. Biochem.* **55**, 913–951
16. Endresen, L., Bakka, A., and Rugstad, H.A. (1983) Increased resistance to chlorambucil in cultured cells with a high concentration of cytoplasmic metallothionein. *Cancer Res.* **43**, 2918–2926
17. Albores, A., Koropatnick, J., Cherian, M.G., and Zelazowski, A.J. (1992) Arsenic induces and enhances rat hepatic metallothionein production in vivo. *Chem. Biol. Interact.* **85**, 127–140
18. Kreppel, H., Liu, J., Liu, Y., Reichl, F.X., and Klaassen, C.D. (1994) Zinc-induced arsenite tolerance in mice. *Fundam. Appl. Toxicol.* **23**, 32–37
19. Shimizu, M., Hochadel, J.F., Fulmer, B.A., and Waalkes, M.P. (1998) Effect of glutathione depletion and metallothionein gene expression on arsenic-induced cytotoxicity and *c-myc* expression *in vitro*. *Toxicol Sci.* **45**, 204–211
20. Hong, S.-H., Toyama, M., Maret, W., and Murooka, Y. (2001) High yield expression and single step purification of human thionein/metallothionein. *Protein Exp. Purif.* **21**, 243–250
21. Pedersen, A.O. and Jacobsen, J. (1980) Reactivity of the thiol group in human and bovine albumin at pH 3–9, as measured by exchange with 2,2′-dithiodipyridine. *Eur. J. Biochem.* **106**, 291–295
22. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
23. Xiong, Y. and Ru, B. (1997) Purification and characteristics of recombinant mouse metallothionein-I from *Escherichia coli*. *J. Biochem.* **121**, 1102–1106
24. Odawara, F., Kurasaki, M., Suzuki-Kurasaki, M., Oikawa, S., Emoto, T., Yamasaki, F., Arias, A.R.L., and Kojima, Y. (1995) Expression of human metallothionein-2 in *Escherichia coli*: cadmium tolerance of transformed cells. *J. Biochem.* **118**, 1131–1137
25. Cols, N., Romero-Isart, N., Bofill, R., Capdevila, M., Gonzàlez-Duarte, P., Gonzàlez-Duarte, R., and Atrian, S. (1999) In vivo copper- and cadmium-binding ability of mammalian metallothionein beta domain. *Protein Eng.* **12**, 265–269
26. Capdevila, M., Cols, N., Romero-Isart, N., Gonzàlez-Duarte, R., Atrian, S., and Gonzàlez-Duarte, P. (1997) Recombinant synthesis of mouse Zn3-beta and Zn4-alpha metallothionein 1 domains and characterization of their cadmium(II) binding capacity. *Cell. Mol. Life Sci.* **53**, 681–688
27. Chen, C.L. and Whanger, P.D. (1994) Interaction of selenium and arsenic with metallothionein: effect of vitamin B12. *J. Inorg. Biochem.* **54**, 267–276
28. Nielson, K.B. and Winge, D.R. (1984) Preferential binding of copper to the beta domain of metallothionein. *J. Biol. Chem.* **259**, 4941–4946
29. Nielson, K.B., Atkin, C.L., and Winge, D.R. (1985) Distinct metal-binding configurations in metallothionein. *J. Biol. Chem.* **260**, 5342–5350
30. Karl, H.S. and Dominik, K. (1993) Quantification of metallothionein in biological materials in *Metallothionein III, Biological Roles and Medical Implications* (Suzuki, K.T., Imura, N., and Kimura, M., eds.) pp. 75–86, Birkhäuser Verlag, Basel, Switzerland
31. Pattanaik, A., Bachowski, G., Laib, J., Lemkuil, D., Frank Shaw III, C., Petering, D.H., Hitchcock, A., and Saryan, L. (1992) Properties of the reaction of cis-dichlorodiammineplatinum(II) with metallothionein. *J. Biol. Chem.* **267**, 16121–16128
32. Lemkuil, D.C., Nettesheim, D., Shaw, C.F., 3rd, and Petering, D.H. (1994) Reaction of Cd7-metallothionein with cis-dichlorodiammine platinum(II). *J. Biol. Chem.* **269**, 24792–24797
33. Vallee, B.L. and Maret, W. (1993) The functional potential and potential functions of metallothionein: A personal perspective in *Metallothionein III, Biological Roles and Medical Implications* (Suzuki, K.T., Imura, N., and Kimura, M., eds.) pp. 1–27, Birkhäuser Verlag, Basel, Switzerland
34. Aopshian, H.V. (1989) Biochemical toxicology of arsenic. *Rev. Biochem. Toxicol.* **10**, 265–300
35. Sunderman, F.W. (1979) Mechanism of metal carcinogenesis. *Biol. Trace Elem. Res.* **1**, 53–86
36. Stillman, M.J., Green, A.R., Gui, Z., Fowle, D., and Presta, P.A. (1999) Circular dichroism emission, and exafs studies of Ag(I), Cd(I), and Hg(II) binding to metallothioneins and modeling the metal binding site in *Metallothionein IV* (Klaassen, C.D., ed.) pp. 23–35, Birkhäuser Verlag, Basel, Boston, Berlin
37. Laib, J.E., Shaw, C.F., 3rd, Petering, D.H., Eidsness, M.K., Elder, R.C., and Garvey, J.S. (1985) Formation and characterization of aurothioneins: Au,Zn,Cd-thionein, Au,Cd-thionein, and (thiomalato-Au)chi-thionein. *Biochemistry* **24**, 1977–1986
38. Sun, H., Li, H., Harvey, I., and Sadler, P.J. (1999) Interactions of bismuth complexes with metallothionein(II). *J. Biol. Chem.* **274**, 29094–29101
39. Scott, N., Hatlelid, K.M., MacKenzie, N.E., and Carter, D.E. (1993) Reaction of arsenic(III) and arsenic(V) species with glutathione. *Chem. Res. Toxicol.* **6**, 102–106
40. Schmöger, M.E.V., Oven, M., and Grill, E. (2000) Detoxification of arsenic by phytochelatins in plants. *Plant Physiol.* **122**, 793–801