

Arsenic binding to *Fucus vesiculosus* metallothionein[☆]

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Abstract

The seaweed *Fucus vesiculosus* is a member of the brown algae family. Kille and co-workers [Biochem. J. 338 (1999) 553] reported that this species contains the gene for metallothionein. Metallothionein is a metalloprotein having low molecular weight, and high cysteine content, which binds a range of metals. *F. vesiculosus* bioaccumulates arsenic from the aquatic environment [Mar. Chem. 18 (1986) 321]. In this paper we describe arsenic binding to *F. vesiculosus* metallothionein, characterized by electrospray ionization mass spectrometry. Five arsenic-MT species were detected with increasing As to protein ratios. These results provide important information about the metal-chelation behaviour of this novel algal metallothionein which is a putative model for arsenic binding to *F. vesiculosus* in vivo. © 2004 Elsevier Inc. All rights reserved.

Keywords: Arsenic; Metallothionein; *Fucus vesiculosus*; Algae; Electrospray ionization mass spectrometry; Heavy metals

The amount of arsenic entering the aquatic environment has increased substantially over the last thirty years and is now a primary concern in many countries [1]. Large amounts of arsenic in various chemical forms and oxidation states are released into freshwater and marine environments through natural erosion processes, smelters, application of herbicides and pesticides, geothermal wells, hot-springs, mining operations, and fossil fuel technologies [2]. The most prevalent arsenic compound in industry today is arsenous oxide (As₂O₃) [3]. It has been reported that As⁵⁺ is biotransformed to As³⁺ in the alga *Fucus gardneri* [4].

The environmental consequences and ultimate fate of arsenic and its different complexes are of great concern and are the subject of considerable research [5]. Brown algae contain higher amounts of arsenic than green or red algae [6]. *Fucus vesiculosus*, which is a brown algae, is found and thrives in heavily polluted aquatic environments [7]. Brown alga *Fucus gardneri* cells have been shown to accumulate arsenic [8]. It has also previously

been shown that arsenic is able to induce metallothionein synthesis [9–12].

Metallothioneins are a family of ubiquitous proteins that are characterized by low molecular weight, high cysteine, and metal content. These proteins sequester not only the essential metal ions zinc and copper but also the toxic heavy metals cadmium and mercury [13–15]. MT is expressed in many vertebrates, fungi, plants, algae, and metal-resistant bacteria [13–16]. In MTs studied to date, metal chelation is achieved through the abundance of cysteine residues with conserved motifs of cys-x-cys, cys-x-x-cys, and cys-cys [17].

While the *F. vesiculosus* MT (O96717) gene has been identified and recombinant protein prepared [7], the metal-binding properties are unknown. We describe in this current work arsenic binding to *F. vesiculosus* metallothionein which could be an important factor in arsenic uptake by this alga.

Materials and methods

Materials. Chemicals used were: CdSO₄, (Fisher Scientific), tetrakis(acetonitrile)copper(I) hexafluorophosphate (Aldrich Chemical) recrystallized from acetonitrile, and 5 mL; arsenic trioxide (AnalaR).

[☆] Abbreviations: MT, metallothionein; fMT, *Fucus vesiculosus* metallothionein; TOF-MS, time of flight mass spectrometry.

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All chemicals used in this study were of the highest grade purity from commercial sources. Hi Trap SP ion exchange columns (Pharmacia) and G-25 Sephadex (Pharmacia) were used in the purification steps.

Protein preparation. The recombinant *Fucus vesiculosus* metallothionein (fMT) protein used in this study was based on the 67-residue sequence: MAGTGCKIWE DCKCGAACSC GDSCTCGTVK KGTTSRAGAG CPCGPKCKCT GQGSCNCVKD DCCGCGK. There are 16 cysteine residues and no disulphide bonds in this sequence. In addition to the sequence from the fMT, the expression system includes the amino acid residues of the stabilizing S-peptide tag (MKETAAAKFE RQHMDSPDLG TLVPRGS) on the N-terminus of the fragment [7]. The recombinant proteins were produced from over-expression of the fusion protein in *Escherichia coli* grown in fresh media containing 50 μ M CdSO₄. Expression was induced with isopropyl- β -D-thiogalactopyranoside (IPTG). The CdSO₄ concentration was increased hourly in the broth over 4 h to a final concentration of 150 μ M. The cells were harvested by centrifugation, resuspended with a 10 mM Tris buffer, pH 7.4, lysed by French press, and centrifuged. The supernatant was applied to an SP ion exchange cartridge, washed with argon saturated 10 mM Tris buffer, pH 7.40, made with deionized water. The metallothionein was eluted with a gradient from a 95% 10 mM Tris and 5% 10 mM Tris-1M NaCl solution. Fractions exhibiting strong absorption at 250 nm, which is indicative of the S \rightarrow Cd charge transfer bands, were pooled.

Further protein purification for MS studies. The protein fractions from the SP cartridge were desalted on a superfine G-25 Sephadex column (100 cm), eluted with an argon saturated 25 mM ammonium-formate buffer with a pH of 7.40. The fractions eluted exhibited the Cd-thiolate band absorption at 250 nm. Fractions containing cadmium bound to the fMT were concentrated on a 200 mL stirred cell concentrator (Millipore) with a 5000 molecular weight cut-off filter. The total amino acid content was confirmed using the mass of the metal-free protein from the ESI-MS experiment. The metal-free fMT was made by eluting the Cd-bound MT on a G-25 column at pH 2.4 with 25 mM Tris buffer. The fractions were confirmed by the UV-vis spectroscopy at a wavelength of 210 nm, which is characteristic of apo-metallothioneins.

Arsenic binding. The arsenic solution used had a concentration of 5.0 mM As³⁺. This was made by dissolving As₂O₃ in concentrated HCl and then raising the pH of the solution to 1.5 with concentrated NH₄OH and argon saturated. Mole equivalent aliquots of this As³⁺ solution were added to the metal-free fMT, the pH raised to 3.0 with 10% NH₄OH, and argon saturated. The arsenic-protein solutions were incubated in a constant temperature water bath at 40 °C for 20 min prior to analysis on the mass spectrometer.

ESI-TOF-MS parameters. The ESI-TOF (Micromass, Montreal, Canada) was calibrated using a solution of NaI and CsI. The scan conditions for the spectrometer were: capillary 3000.0 V, sample cone 50.0 V, RF lens 1126.0 V, extraction cone 5.0 V, desolvation

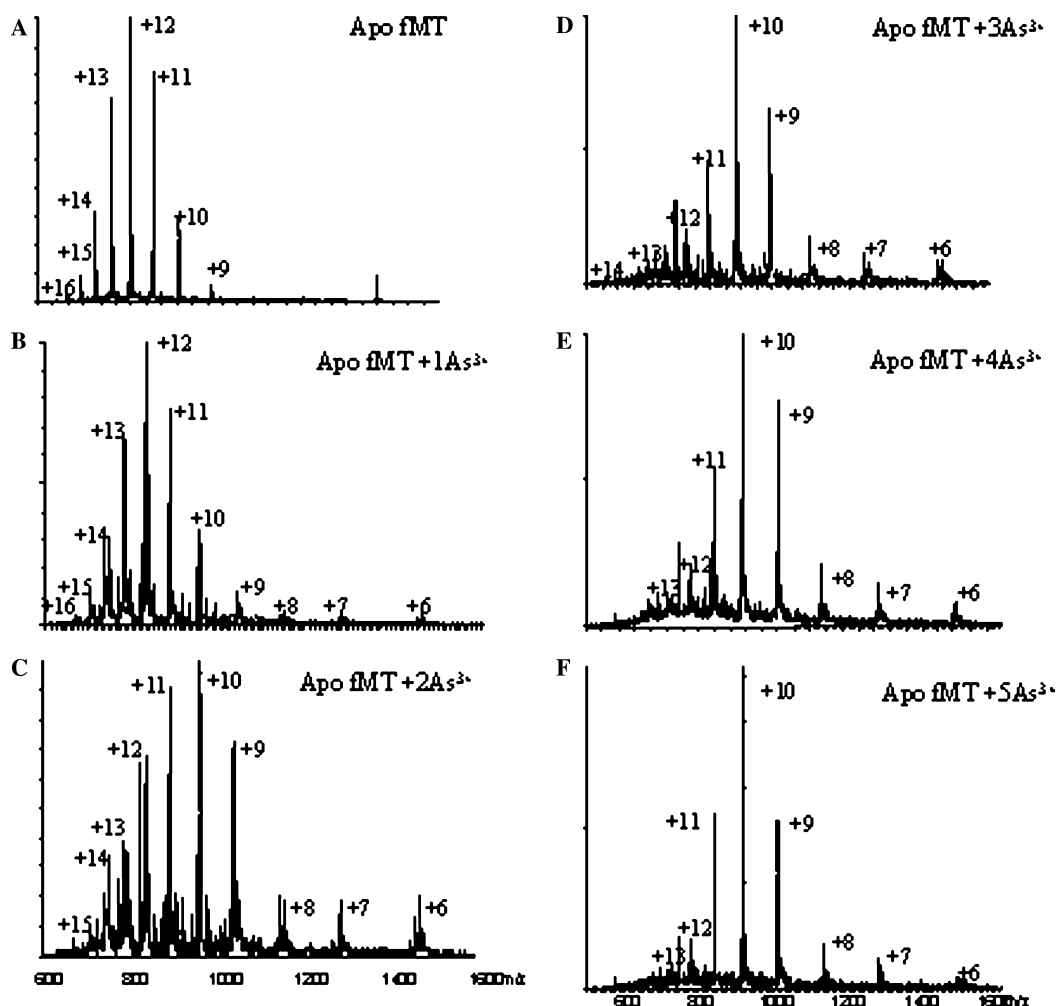


Fig. 1. ESI mass spectra from the analysis of solutions containing apo fMT and increasing molar equivalents of As^{III} (A, no As^{III}; B, 1 As^{III}; C, 2 As^{III}; D, 3 As^{III}; E, 4 As^{III}; and F, 5 As^{III}). The numbers labeled on the peaks are the observed charged species for the reconstructed parent ions.

temperature. 20 °C, source temperature 80 °C, cone gas flow 63 L/h, and desolvation gas flow 545 L/h. The m/z range was 500.0 to 2500.0, the scan mode was continuum, and interscan delay was 0.10 s. The observed spectra were reconstructed using the Max Ent program from Mass Lynx software package.

Results

We report the mass spectra recorded for the addition of 1 to 100 mol equivalents of As^{3+} . In Fig. 1, we show the observed charge states as a function of increasing concentration of added As^{3+} to metal-free (apo) fMT. For metal-free fMT at pH 3.0, Fig. 1A, the charge states correlate to: +16 (m/z 597.02), +15 (m/z 637.01), +14 (m/z 682.49), +13 (m/z 734.91), +12 (m/z 796.10), +11 (m/z 868.35), +10 (m/z 955.12), and +9 (m/z 1061.12). The predominant charge state is the +12 charge state. While the +15 and +16 charge states can be located in the mass spectrum, their intensities are so low that these species represent fractions of less than 1–2% of the total and are not significant. The highest meaningful charge state is the +14, which implies complete protonation of all 14 basic amino acid residues identified below. From the sequence below we can identify fourteen basic amino

acids present in this peptide chain that are available for protonation at low pH (underlined in the sequence). S-tag: MKETAAAKFERQHMDSPDLG TL VPRGS and fMT: MAGTGCKIWEDCKCGAACSCGDSC TCGTVKKGTTSRAGAGCPCGPKCKCTGQGSCN CVKD DCCGCGK. The unusual presence of the +6 state (1591 a.m.u.) seen in Fig. 1A represents a small fraction of a much more folded conformation, which only disappears at the fully metallated state, Fig. 1E.

Addition of 1 and 2 molar aliquots of As^{3+} (Figs. 1B and C) results in the overlay of the observed charge states from two series, one with +12 as the maximum, the other with a +10 maximum. The reconstructed spectra, Fig. 2, show that As_n -fMT species with $n = 1, 2, 3$, and 4 are being formed.

Addition of 4 and 5 As^{3+} results in a single species, As_5 -fMT, forming with a single series of charge states, which exhibit a maximum at +10, Fig. 1F. The observed charge states are: +11 (m/z 901.25), +10 (m/z 991.30), +9 (m/z 1101.23), +8 (m/z 1238.74), +7 (1415.55), and +6 (m/z 1657.58).

The reconstructed spectra for each of the sets of charge states are shown in Fig. 2. For the apo-fMT (Fig. 2A) there is only the metal-free fMT (9517.60 a.m.u.) present. For the addition of one molar

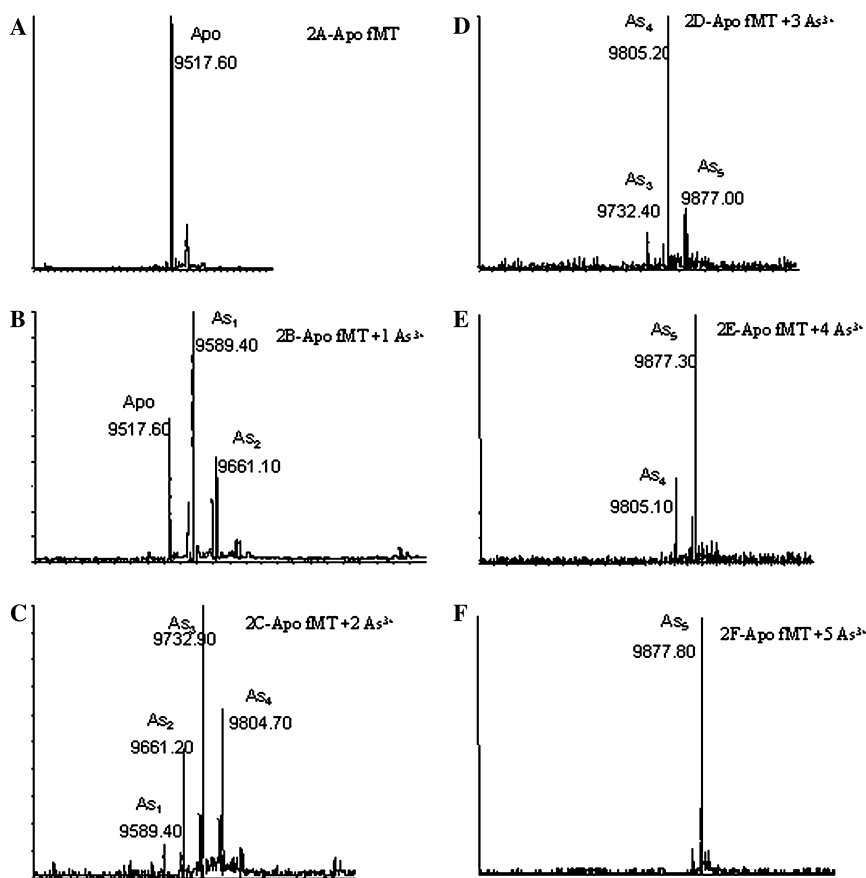


Fig. 2. Reconstructed spectra from the observed charge states of the spectra obtained from the addition of increasing molar equivalents of As^{III} (A, no As^{III} ; B, 1 As^{III} ; C, 2 As^{III} ; D, 3 As^{III} ; E, 4 As^{III} ; and F, 5 As^{III}). The spectra were reconstructed using the Max Ent program from Micromass.

equivalent of As^{3+} (Fig. 2B) there are three species: apo-fMT (9517.60 a.m.u.), As_1 -fMT (9589.40 a.m.u.), and As_2 -fMT (9661.0 a.m.u.). Addition of two molar equivalents of As^{3+} (Fig. 2C) results in As_1 -fMT (9589.20 a.m.u.), As_2 -fMT (9661.20 a.m.u.), As_3 -fMT (9732.90 a.m.u.), and As_4 -fMT (9804.70 a.m.u.). Only following the addition of five molar equivalents of As^{3+} to apo fMT (Fig. 2F) is the single As_5 -fMT (9877.80 a.m.u.) observed. Further addition of excess As^{3+} solution up to a ratio of greater than 100 As to 1 MT did not result in further binding to the fMT.

Discussion

Arsenic is characterized as a human carcinogen. High levels of arsenic in drinking water have been associated with a prevalence of skin, lung, and bladder cancers [18]. In solution, arsenic (as As^{3+}) binds readily to sulphhydryl groups [19], which is considered to be the origin of its toxicity. Once arsenic is bound directly to proteins it inhibits their functions [20].

Bioaccumulation of arsenic in algal species leads to its incorporation into the food chain of higher species, which is an area of great concern [21–23].

The seaweed species *F. vesiculosus* sequesters large amounts of heavy metals from the aquatic environment and binds them in sulphur-rich vesicles termed physodes [24]. Heavy metals are concentrated from water in *F. vesiculosus* by a factor of between 10^3 and 10^4 [25]. Reports of *F. vesiculosus* functioning as a biomonitor of metal ions in the aquatic environment lead to the question of the molecular chelating agent and metal-binding mechanism for the heavy metals. Metallothionein is a clear contender due to the high ratio of cysteines in the sequence and the known metal-binding properties [26,27]. However, the metallothionein gene has only recently been identified for this seaweed species by Kille and co-workers [7], and arsenic binding has only recently been demonstrated to mammalian metallothioneins, the human and rabbit liver species [20,28]. While a single role for MT has not yet been determined, the range of metals chelated suggests involvement in transport, metabolism, and detoxification of metals [13–15].

We show in Fig. 1 the observed charge states for a series of As-containing metallothionein species formed upon the addition of increasing molar equivalents of arsenic to metal-free fMT. Because the charge states represent exposed, basic amino acids, we can associate the number of charge states observed with the exposure of residues to the solvent and deduce possible conformational information. The predominant species is the +12 charge state for the apo-fMT and As_1 -fMT. As As^{3+} is added, a change in the distribution of charge states becomes noticeable (Fig. 1C). By the addition of two molar equivalents of As^{3+} to form a mixture of

As_1 , As_2 , As_3 , and As_4 , the predominant charge state is the +10. We can see that the charge states in Fig. 1C reflect the mixture of a +12 maximum for As_1 , As_2 , and As_3 , and a +10 maximum for As_4 , by examining the spectral data shown in Fig. 1D, which is associated with formation of almost pure As_4 -fMT (Fig. 2D). The +10 maximum charge state remains up to the saturation point of As_5 -fMT in Fig. 1F. This change from +12 for the apo-fMT and first 3 bound As and +10 for the saturated As_5 -fMT represents loss of access to basic amino acids. Not only does the equilibrium protonation point change (to +10) but also the +11 charge state is the extreme of the equilibrium mixture at this point, compared with the +14 of the apo-fMT. Significantly, the Cd_6 -fMT and Zn_6 -fMT species exhibit a maximum charge state of +7 with no significant counts above +8 [29]. We interpret these observations in terms of the structures that form during metallation of fMT with the three metals, As, Cd, and Zn, below.

The characterization of the charge states of metallothionein at pH extremes have been previously studied [30]. Charge states observed as Cu(I) binds to the α and β domains of human metallothionein have been characterized using ESI-MS [31].

Reconstructed spectra

The reconstructed spectrum is shown for the metal-free fMT and the As metallated species in Fig. 2. Additions of increasing concentrations of As result in formation of As_5 -fMT as a single species. No other ligands are involved in the As binding.

Assuming that the As^{3+} binds according to its usual inorganic chemistry [20], each trivalent arsenic atom can bind up to three cysteinyl sulphurs in a trigonal pyramidal configuration. For the As_5 -fMT species, this would require 15 cysteines coordinated to the 5 As^{3+} and one free proton for the 16th cysteine, exactly as measured.

Structural aspects of As binding to fMT

The fMT sequence (above) suggests that there may be two metal-binding domains, the smaller, a 7 cysteine region (7-cys) separated by a 14 residue linker region from the larger 9-cysteine region (9-cys). For Cd and Zn, we expect a cluster structure in which bridging and terminal sulphurs from the 16 cysteines will form a cage-like, or adamantane-like, three-dimensional structure. In this case, the difference in the number of charge states observed in the mass spectrum between the open metal-free, apo-fMT, and closed metallated M_6 -fMT is now the number of basic amino acids that are blocked from the solvent and remain unprotonated. Metallation with Zn and Cd requires four thiols arranged in tetrahedral geometry around the six divalent metals. In the case of As^{3+} , the metallation requires just three thiols with the fourth

position occupied by a nonbonding pair. The transition only to the +10 charge state upon metallation to the 5 As state, and the presence of the +11 state, together suggest that there is a much more open structure in the As-containing fMT than in the Cd or Zn structures.

Although no structures are available for As-containing metallothioneins, we can discuss the structural properties identified by the mass spectral charge states. The As appears to bind in two steps: (i) up to three As with a change in charge states from a +12 maximum to a +10 maximum, and (ii) further As up to 4–5, with no change in charge states. We can suggest that the first three As bind to the 9-cys region to form As₃Cys₉—with a certain degree of folding that blocks the 3 amino acid residues from protonation, the 4th and 5th As bind to the 7-cys region to form As₂Cys₆Hcys₁.

Conclusions

Trivalent arsenic binding to metallothionein from the seaweed *F. vesiculosus* is reported for the first time. Five As³⁺ atoms bind to the protein. The mass spectral data suggest that the structure is far more open than that observed when six divalent metal ions bind to the fMT.

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