HPLC/ICP Mass Spectrometric Study of the Selenium Incorporation into Cyanobacterial Metallothionein Induced under Heavy-Metal Stress

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Selenium is a toxic element which can cause environmental pollution. Its uptake and incorporation by Zn²⁺-induced metallothionein (MT) biosynthesis in a cyanobacterium were studied by means of high-performance liquid chromatography (HPLC) with inductively coupled plasma mass spectrometric (ICPMS) detection. When we cultured the cells in selenite-enriched media, as much as 60% of the total selenium in the water-soluble cell components was incorporated into MT, which exists as two major isoforms but occupies a very minor fraction of the overall proteins. Existence of two pathways has been shown for the first time for selenium incorporation into the cyanobacterial MT; one is the S-Se bonding which can be reductively broken by 2-mercaptoethanol, and the other is the formation of selenocysteine followed by its incorporation into the MT peptide chain. No essential difference was noted between selenite and selenate regarding the uptake or the incorporation of Se. The present HPLC/ICPMS system can elucidate biological processes of environmental chemical importance at the molecular level.

Keywords Inductively coupled plasma mass spectrometry, high-performance liquid chromatography, metallothionein, isoform, zinc, selenium, selenocysteine, cyanobacterium, heavy metal

Metallothioneins (MTs) are a class of low molecular weight, cysteine-rich, metal-binding proteins found in various organs of animals, plants, eukaryotic microorganisms and prokaryotes including cyanobacteria (blue-green algae).¹ MTs are induced when such heavy metal ions as Cu²⁺, Zn²⁺ and Cd²⁺ are administered in high amounts. Contamination of aquatic microorganisms with heavy metals is one of the major concerns in environmental chemistry and biochemistry since they are, as accumulators of heavy metals, the primary food which enters trophic chains in the aquatic system. Cyanobacteria can accumulate heavy metals to a concentration several orders-of-magnitude higher than in the surrounding medium. Most of the incorporated excess heavy metals are converted into MT.

Selenium, though toxic when dosed in high amounts^{2,3}, is an essential nutrient that is taken up in several different chemical forms. The bioavailability and metabolism of different chemical species of Se in the diet are receiving much attention in nutrition science. Dietary Se in the form of amino acids such as selenomethionine and selenocysteine is more readily absorbed than inorganic selenite and selenate.⁴ At the present time, when environmental pollution by selenium used in various advanced devices is becoming of increasing concern, its fate analysis in the biosphere is also of much importance.

Although selenium itself does not trigger the biosynthesis of MT, there are a few reports indicating the association of Se with mammalian MTs by Cd-induced

synthesis of MT.⁵ In some higher plants, uptake of selenate or selenite leads to the synthesis of amino acids, selenocysteine or selenomethionine, in a manner similar to sulfur metabolism.³ The toxicity of Se to plant species supposedly arises from indiscriminate incorporation of Se in place of S into S-containing amino acids of cellular constituents, since organoselenium compounds are generally less stable and more reactive than the sulfur counterparts.⁶ However, it still remains unclear whether selenocysteine does actually enter the polypeptide chains. To our knowledge, no report has unambiguously identified specific protein(s) with which selenium is associated in plants or microorganisms.

For the speciation of cyanobacterial MT, we have developed a measurement system by combining high-performance liquid chromatography (HPLC) with inductively coupled plasma mass spectrometry (ICPMS).⁷ In the present work, this technique is applied to the examination of *in vivo* selenium association with cyanobacterial MT, especially the identification of the chemical form(s) of Se in MT, by use of size exclusion (SE)-, reversed-phase (RP)- and cation exchange (CE)-HPLC.

Experimental

Materials

Seleno-D,L-cystine and seleno-L-methionine were purchased from Sigma; dithioerythritol (DTE), 2-

mercaptoethanol and iodoacetic acid were purchased from Wako Pure Chemical Industries; and dithiothreitol (DTT) was purchased from Tokyo Kasei Kogyo. All were used without further purification. Metal standard solutions were prepared by diluting and mixing commercial standard solutions for AAS (Wako Pure Chemical Industries). Water deionized with a Millipore Milli-Q system was used throughout.

Culture conditions

A cyanobacterium *Anacystis nidulans* R-2 was grown in a 3-L of Cu-depleted BG-11 medium⁸ with 10 μ M of ZnCl_2 and 30 μ M of Na_2SeO_3 added at 23°C under fluorescent lamps (ca. 1000 lx). In additional experiments, Na_2SeO_4 was employed in place of Na_2SeO_3 . Cells were harvested after culturing for about 3 weeks, until the growth reached a stationary phase.

Sample preparation

The cells harvested by centrifugation were washed three times with 10 ml of a 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, to remove metal ions reversibly bound to the cell wall. Then they were homogenized by ultrasonification in 2 ml of a cold 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM dithiothreitol (DTT). The homogenate was then centrifuged at 143000 $\times g$ for 1 h at 4°C; the resulting supernatant was submitted to the SE-HPLC/ICPMS measurements for the speciation of MT.

Fractionation of cytosolic proteins

The supernatant was separately chromatographed on a preparative-scale Sephadex G-75 column (2.6 $\times$ 75 cm). The elution buffer was 10 mM NH_4HCO_3 containing 5 mM 2-mercaptoethanol, with a flow rate of 1.0 ml/min and a fraction volume of 3.15 ml. The fractions were monitored for absorbance at 254 nm, and the concentrations of Zn and Se in each fraction were determined by ICPMS. The fractions corresponding to the molecular weight of about 8000 and containing Zn and Se were collected and lyophilized for the speciation of MT isoforms and selenium species.

Speciation of selenium species

Carboxymethylation of the MT fraction was performed with iodoacetic acid.⁹ One milligram of the lyophilized sample was dissolved in 50 μ l of 0.2 M Tris-HCl buffer (pH 8.8) containing 8% (w/v) sodium dodecyl sulfate (SDS), 50% (v/v) glycerol and 5 μ l of 0.2 M DTE; the mixture was then boiled for 5 min. After the reaction mixture was cooled, 7 μ l of a 1 M iodoacetic acid solution of pH adjusted to about 8 with NaOH just before use, was added to the mixture, followed by incubation at 50°C for 15 min. The reaction mixture was then dialyzed against water and lyophilized to obtain the derivatized proteins for hydrolysis.

The lyophilized sample was re-suspended in 6 M HCl for hydrolysis. An aliquot of the acid suspension was

Table 1 Chromatographic conditions and ICPMS operating procedures

	Separation of MT	Separation of MT isoforms	Separation of Se species
Chromatographic conditions			
Column	Size exclusion (SE) Asahipak GFA-30F (Asahi Chem. Ind.) 7.6 mm i.d. $\times$ 300 mm	Reversed phase (RP) Capcellpak C ₈ (Shiseido) 4.5 mm i.d. $\times$ 150 mm	Cation exchange (CE) Nucleosil 100-5SA (M. Nagel) 4.6 mm i.d. $\times$ 250 mm
Mobile phase	50 mM Tris-HCl (pH 7.5) +0.2 M $(\text{NH}_4)_2\text{SO}_4$, 0.1 mM EDTA	9:91 (v/v) acetonitrile: 50 mM Tris-HCl (pH=7.5) +0.1 mM EDTA	A: 25 mM $\text{NH}_4\text{H}_2\text{PO}_4$ (pH 3.0) B: 25 mM $(\text{NH}_4)_2\text{HPO}_4$ (pH 7.0)
Elution profile	isocratic	isocratic	linear gradient: 0–20 min: 0–67% B
Flow rate (ml min ⁻¹)	0.8	1.0	1.0
Injection volume (μ l)	20	20	20
ICPMS operating procedures			
Argon gas flow rate (1 min ⁻¹)			
outer	18.0	17.5	18.0
auxiliary	1.0	1.6	1.0
nebulizer	1.2	0.90	1.2
O ₂ fraction in nebulizer	0	0.08	0
Forward power (kW)	1.2	1.5	1.2
Isotopes monitored	⁶⁸ Zn, ⁷⁸ Se	⁶⁴ Zn, ⁷⁸ Se	⁶⁴ Zn, ⁷⁸ Se

sealed in a glass ampoule under reduced pressure after repeated evacuation. The sealed ampoule was heated for 40 h at 110°C, then opened; the contents were dried *in vacuo*. The hydrolysate was re-dissolved in 50 mM Tris-HCl buffer (pH 7.0) for ion exchange chromatography.

Carboxymethylated (CM-) selenocysteine standard was prepared by the method of Brown and Shrift.¹⁰ Ten milligrams of selenocystine was suspended in 3 ml Tris-HCl (pH 8.6), to which 0.1 ml of 2-mercaptoethanol was added to reduce the selenocystine into selenocysteine at room temperature for 4 h under N₂. Selenocysteine was then carboxymethylated by addition of iodoacetic acid (500 mg) dissolved in 1 ml of 1 M NaOH, followed by incubation at room temperature for 15 min under N₂. The mixture was applied with the MT hydrolysate to CE-HPLC/ICPMS for the assay of selenocysteine in MT.

HPLC/ICPMS measurements

The HPLC system was composed of a JASCO 880-PU dual-piston HPLC pump, a Rheodyne 7125 injector with a 20- μ l injection loop, and a Rheodyne 7030 switching valve and an appropriate analytical column. The chromatographic conditions are summarized in Table 1. For SE-HPLC, 0.2 M (NH₄)₂SO₄ was added in the eluent to attain quantitative protein recovery. For SE- and RP-HPLC, 0.1 mM EDTA was added in the eluent to prevent the adhesion of inorganic metal ions to the separation gel, such adhesion tends to generate anomalous metal peaks in subsequent runs. In the CE-HPLC/ICPMS measurement, changes in the sensitivity of ICPMS caused by the pH-gradient elution during chromatographic runs were less than 5%; this was comparable to the value obtained under isocratic elution. Hence no sensitivity corrections were made on any measurements.

The ICPMS apparatus used was SPQ-6100 (Seiko Instruments Inc.). Table 1 summarizes the instrumental operating conditions of ICPMS for each mode of measurement. The chromatogram peak areas and retention times were determined by use of the SPQ-6100 software and an NEC PC-9801 computer. The choice of the third abundant Zn isotope mass number ($m/z=68$), instead of the first ($m/z=64$) and second ($m/z=66$) abundant isotopes, in the SE-HPLC/ICPMS operation is because of severe interferences from polyatomic ions S₂⁺ and SO₂⁺ produced in the argon plasma from SO₄²⁻ in the eluent. Similarly, the second abundant mass number of 78 was chosen for the Se monitoring since the most abundant isotope ($m/z=80$) undergoes interference from argon dimer ions Ar₂⁺.

HPLC/ICPMS determination of elements was achieved *via* peak-area calibration using metal standard solutions. By controlling the switching valve located between the injection valve and the HPLC, the system can be operated in a flow-injection (FI) mode (FI/ICPMS). This facilitated quantitation of samples and standards under the same flow conditions as for the HPLC column. Thus, as soon as the sample elution and

Fig. 1 SE-HPLC/ICPMS chromatograms for the water-soluble fraction of cyanobacterium (*Anacystis nidulans* R-2) cultured in the BG-11 medium containing ZnCl₂ (10 μ M) and Na₂SeO₃ (30 μ M).

ICPMS detection were completed within about 900 s (SE- and CE-HPLC) or 1200 s (RP-HPLC), the system was changed to the FI/ICPMS mode and a standard Zn and Se solution was injected for metal quantitation. A batch measurement was complete within 1200 s (SE- and CE-HPLC) or 1500 s (RP-HPLC).

Results and Discussion

Speciation of MT by SE-HPLC/ICPMS

Figure 1 shows the SE-HPLC/ICPMS chromatograms, monitored at mass numbers of Zn (68) and Se (78), for the water-soluble fraction of cyanobacterial homogenates. The peaks at 1000 s are 1-ppm standard Zn and Se peaks detected in the FI/ICPMS mode. The peaks at a retention time of 580 s are assignable to the Zn-MT induced in the cyanobacterium.^{7c} Clearly, Zn is associated exclusively with the MT fraction. On the other hand, there are two major Se peaks in the elution profile. One is eluted just after the column void volume (435 s) associated with large molecular weight proteins, and the other is eluted exactly at the peak of MT. Selenium in the latter peak represented 58% of the total Se that was eluted from this column. A minor Se peak at 740 s is an unidentified, low molecular weight Se compound.

Figure 2 shows the Sephadex G-75 elution profiles of the same sample as for Fig. 1. Fractions in the elution volume of 100–232 and 232–279 ml were separately collected and lyophilized. The weight of the former

Fig. 2 Sephadex G-75 elution profiles for the water-soluble fraction of cyanobacterium. See text for details.

Fig. 3 RP-HPLC/ICPMS chromatograms for the MT fraction obtained by Sephadex G-75 gel filtration of the water-soluble fraction of cyanobacterium.

fraction was 31.1, and that of the latter was 3.0 mg. The fraction in the elution volume of 232–279 ml essentially consists of MT, which is barely detectable in the UV

absorbance chromatogram. From these data, the Se/protein ratio for MT was calculated to be roughly 14 times higher than that for other, heavier proteins: this shows the selective incorporation of Se into MT. The MT fraction was stored for further analysis.

The relative intensity of the lower molecular weight band (320–400 ml) is much stronger in the Se-chromatogram of Fig. 2 than that in the Se-chromatogram in Fig. 1: the fractions of the lower molecular weight component are 9 and 36% in Figs. 1 and 2, respectively. This is suggestive of a release of Se from the MT fraction during separation in the Sephadex G-75 column. A portion of Se has probably been covalently linked to the sulfhydryl groups of cysteine residues in MT, and 5 mM 2-mercaptoethanol in the eluent reductively cleaved the S–Se bond. If this is indeed the case, the fraction of Se bound to MT via the S–Se bond is estimated to be $36 - 9 = 27\%$. The existence of S–Se type bonding was demonstrated by Weser *et al.* by X-ray photoelectron spectroscopy *in vitro*.¹¹ They reported that a selenotrisulfide was formed when selenite was incubated with a Zn-MT apoprotein prepared from rat liver.

Speciation of MT isoforms by RP-HPLC/ICPMS

Reversed-phase HPLC is a powerful means for the separation of MT isoforms, because it can even effectively separate peptides that differ in their composition only by a single amino acid.¹² When the cyanobacterial MT fraction collected as described above was submitted to separation and detection by RP-HPLC/ICPMS, two major MT peaks were detected, as displayed in Fig. 3. After carboxymethylation following SDS-PAGE, each of these peaks gave a single homogeneous band on the gel by silver staining (data not shown). These two MT isoforms exhibited different Se and Zn binding properties. Thus, the Se/Zn molar ratios of the forms at retention times of 300 and 450 s were 0.07 and 0.04, respectively. The molecular level picture underlying such a difference is not known at present, and is to be elucidated in future work.

Olafson *et al.* reported that a Cd-MT from another cyanobacterial strain (*Synechococcus* strain TX-20) was separated into seven isoforms by reversed-phase column chromatography.¹³ They attributed the large number of separable MT forms to the nature of metals incorporated therein and to the degree of metal saturation in MT. Since a single metal ion (Zn^{2+}) was used for the MT induction and hence Zn was the sole metal incorporated into MT in the present work, the two MT isoforms probably differ in the amino acid composition. Thus, the RP-HPLC/ICPMS detection is useful for determining the metal distribution among the MT isoforms as well as the selective accumulation of selenium in a particular isoform.

Speciation of Se species by CE-HPLC/ICPMS

Selenocysteine is highly susceptible to oxidative degradation, because the selenol group has a significantly lower oxidation redox potential than its sulfur counter-

Fig. 4 CE-HPLC/ICPMS chromatograms for the hydrolyzed MT fraction obtained by Sephadex G-75 gel filtration of the water-soluble fraction of cyanobacterium.

part.⁶ To stabilize selenocysteine and prevent its degradation, the carboxymethyl derivative was synthesized by addition of iodoacetic acid to the Zn-MT fraction obtained above. Figure 4 shows a cation exchange (CE) chromatographic elution profile of the hydrolysate of the carboxymethylated MT fraction. The peak assignments were done by adding standard CM-selenocysteine and selenomethionine to the sample. The chromatogram reveals that 46% of Se exists as CM-selenocysteine (A) and 4% as selenomethionine (D). Peak B is assignable to a denatured form of CM-selenocysteine, in view of the observation that standard CM-selenocysteine stored in an acidic solution (0.1 M HCl) was also converted into a species giving peak B. Probably peak B originates in an intramolecularly condensed form of CM-selenocysteine, by analogy with the reaction found for CM-cysteine.¹⁴ The sum of peaks A and B thus reveals that 70% of Se in MT exists in the form of

selenocysteine. Peak C, amounting to 19% of the total Se, remains unidentified. Despite this uncertainty, this result clearly indicates that selenocysteine is the dominant form of the tightly bound selenium in cyanobacterial MT.

Selenate versus selenite for incorporation of Se into MT

Of different chemical forms of selenium usually present in soils, only the inorganic anions, selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}), are known to be toxic to higher plants. This toxicity can be attributed to a combination of three factors: (i) both anions are readily absorbed by roots and translocated to other parts of the plant, (ii) both are metabolized into organoselenium compounds within the cell, and (iii) these metabolites act as analogues of essential sulfur compounds and thereby interfere with biochemical reactions.³

To see if the anionic form affects the mode of Se incorporation into different compounds *in vivo*, the cyanobacterium was cultured in Na_2SeO_4 - and Na_2SeO_3 -containing culture media and the resulting Zn and Se distributions were compared. The results are summarized in Table 2. A slight difference was noted between selenite and selenate in the RP-HPLC/ICPMS chromatograms. When dosed with Na_2SeO_4 , a relatively large portion of Zn and Se was eluted at the void volume. The release of Zn^{2+} from MT entailed the release of Se. This may reflect the existence of Zn-Se-R bonds in which R is any residue that is not bound to the apo-MT, though further study is needed to substantiate this speculation. Except for such a minor difference, no clear difference was noted between SeO_4^{2-} and SeO_3^{2-} regarding the Se-incorporation in MT. This suggests that selenate is reduced into selenite after the uptake by the cyanobacterial cell. We tentatively propose that sulfate-reducing enzymes are responsible for the selenate reduction.³

Though the function of selenocysteine as an integral component of some mammalian and microbial enzymes is well-documented², and the ability of plants to synthesize this selenoamino acid has been demonstrated³, almost nothing is known about the mode of its incorporation into proteins of plants or microorganisms.

Table 2 Content (%) of Se or Zn in three different fractions against the total Se or Zn detected chromatographically, for cyanobacterial cells cultured in the BG-11 medium containing (A) ZnCl_2 (10 μM) and Na_2SeO_3 (30 μM) or (B) ZnCl_2 (10 μM) and Na_2SeO_4 (30 μM)

	SE-HPLC/ICPMS			Sephadex G-75			RP-HPLC/ICPMS			CE-HPLC/ICPMS		
	>MT	MT	<MT	>MT	MT	<MT	v.v. ^a	MT-1	MT-2	Se-Cys	Se-Met	peak C ^b
(A) Se	33	58	9.1	37	27	36	8.7	52	29	70	4.2	19
Zn	N.D.	ca. 100	N.D.	N.D.	85	3	1.3	46	44			
(B) Se	29	63	8.0	37	32	31	38	36	20	74	1.6	13
Zn	N.D.	ca. 100	N.D.	4.6	87	8.4	6.0	58	26			

>MT, larger molecular weight fraction; MT, MT fraction; <MT, lower molecular weight fraction. a. Void volume peak. b. Unidentified species (see Fig. 4).

The present study has unequivocally demonstrated the existence of two pathways for Se incorporation in the cyanobacterial MT: (a) covalent binding of Se to MT as selenocysteine and (b) S-Se type binding which can be broken by the action of 2-mercaptoethanol.

The human body can absorb dietary selenium in the form of such amino acids as selenomethionine and selenocysteine more easily than it can the inorganic selenite and selenate.⁴ We have found here that co-administration of Zn²⁺ and selenite or selenate in cyanobacteria leads to the incorporation of Se in the peptide chain of MT, and selenocysteine is the dominant form of the Se compound. This may be hazardous for biological activities. Toxic elements including heavy metals are accumulated in the environment through human activity.¹⁵ Such biotransformation of Se in microorganisms may cause increased accumulation of Se in edible living organisms. Further work is necessary to clarify such synergistic effects of toxic elements on the biosphere.

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