CROATICA CHEMICA ACTA CCACAA **70** (1) 247–257 (1997)

ISSN–0011-1643
CCA–2419

Original Scientific Paper

Electrochemical Characterization of Metal-Binding Properties of Metallothioneins Isolated from *M. galloprovincialis**

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Received February 15, 1996; accepted July 1, 1996

Amperometric titrations of defined metallothionein concentrations with the standard $CdCl_2$ solution have been performed in seawater samples at pH ≈ 1 and pH ≈ 8, in order to define the metal-binding properties of purified mussel metallothionein component. The concentration of the formed Cd-thionein complex has been assessed in the indirect and direct mode. The results on the available ligand concentration C_L for complexing Cd^{2+} ions and the apparent stability constant K', determined by two procedures are compared and the differences discussed. Complexing characteristics of the metallothionein can be reliably determined only if the evaluation is based on the specific signal of Cd-thionein complex.

INTRODUCTION

Metallothioneins (MTs) are low molar mass metal-binding proteins, known to occur in all animal phyla as well as in fungi, in some plants and in cyanobacteria.[1] The protein was designated as »metallothionein« on account of its exceptionally high metal and sulfur content.[2] Its apoprotein form is assigned as thionein. MTs induced in hepatic and other tissues are specific molecular biomarkers of the exposure of vertebrate and invertebrate species to metals.[3] MTs of mammalian origin are characterized in detail regarding their biological and physico-chemical features, the binding sites, stoichiometry and geometry of metal complexes.[4]

* Dedicated to Marko Branica on the occasion of his 65th birthday.

Sessile and filter-feeding marine organisms which are widely distributed and used as indicators of metal pollution of coastal areas are mussels of the genus *Mytilus*.[3] In the digestive gland of bivalve mussels, MTs are induced as a response to the bioavailable Cd^{2+} concentration. Occurrence of MTs in *Mytilus* sp. has been determined in both laboratory and field studies.[5–9] In contrast to the mammalian type of MTs, the mussel type of MTs is insufficiently characterized. Complete amino-acid sequences of seven isoforms of MTs, isolated from the edible part of the mussel *Mytilus edulis* have been published recently.[10] The mussel MTs exhibit more similarity to the vertebrate MTs than to those of non-moluscan invertebrates. Isoforms of mussel MTs exhibit homology to the mammalian class I MTs.

Due to their sensitivity and selectivity, electrochemical methods have a particular advantage in determination of the MTs and metal content, as well as in speciation studies. In our study the electrochemical method has been applied to the characterization of the dissolved chemical species of cadmium. Since the understanding of the biological function of the proteins is ultimately based on the knowledge of their molecular features and physicochemical properties, the aim of our study has been focused on the characterization of the metal-binding properties of purified MT component, induced and isolated from the digestive gland of *M. galloprovincialis*.

EXPERIMENTAL

Chemicals and Materials

Solutions were prepared from the analytical-reagent grade chemicals and doubly distilled water. For voltammetric measurements Adriatic seawater was used as the supporting electrolyte. The water was sampled at the open marine station in Central Dalmatia (salinity 38‰, pH = 8.3 ± 0.1), according to the procedure for trace metal analysis.[11] The standard Cd^{2+} solution (1.000 ± 0.002 g/L) was prepared from the concentrated Titrisol solution (Merck), which contains $CdCl_2$.

The results presented and discussed in this paper refer to the purified MT II component isolated from the digestive gland of *M. galloprovincialis*. Specimens of *M. galloprovincialis*, 5 to 8 cm shell length, were exposed to 1.8×10^{-6} mol/dm^3 Cd^{2+}, added as $CdCl_2$, for 20 days in an open continuous flow-through seawater system. The composite sample of the digestive gland of cadmium-exposed *M. galloprovincialis* was homogenized in a Tris-HCl buffer pH = 8.6, containing 1 mM dithiotreitol (DTT) and protease inhibitors. The homogenate was centrifuged at 28000 xg and 4 °C for 1 h. The digestive gland supernatant was fractionated by gel-filtration on a Sephadex G–75 chromatographic column (1.6 × 120 cm) using ammonium bicarbonate elution buffer pH = 8.6. Pooled chromatographic fractions of the prevailing MT content were further purified on a diethylamino ethyl (DEAE)-cellulose column (1.6 × 15 cm) and eluted by increasing the concentration gradient of ammonium bicarbonate buffer containing DTT. The elution profiles and other details on the isolation and purification procedures of MT components have been described by Pavičić

et al.[12] Following the purification of the MT fractions from DEAE-cellulose column, electrophoretic mobility patterns, including apparent molar mass estimation, were recorded on a sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS– PAGE). Two MT components were isolated. The more negatively charged MT II component contained a single band of apparent molar mass 12.6 kDa and corresponds to the pure MT protein.

Amperometric Titration of Mussel Type of Metallothionein with Cd^{2+}

Measurements in a differential pulse anodic stripping voltammetric mode (DPASV) were performed in a quartz Metrohm type voltammetric cell at the hanging mercury drop electrode (HMDE). The reference electrode was an Ag/AgCl/sat. KCl electrode connected to the cell *via* a salt bridge filled with genuine seawater. The counter electrode was a platinum wire. Electrochemical deposition of Cd(II) was performed at –0.90 V *vs.* the potential of reference electrode, stirring the solution with a Teflon coated magnetic bar at 800 rpm, to speed up the mass transfer of solution to the electrode. The deposition time was 120 s followed by a 30 s resting time without stirring. The instrumental set-up for DPASV mode was: voltage pulse 50 mV amplitude and 57 ms duration with a clock time of 0.5 s and a scan rate of 5 mV/s. The amalgam formed during the deposition step at the HMDE surface was reoxidized during the anodic potential shift. Voltammetric measurements were performed at a constant temperature of (25.0 ± 0.2) °C. Before each measurement the solution was thoroughly deaerated with a stream of extra pure nitrogen, and during the measurement, a stream of nitrogen was passed over the surface of measuring solution. Before entering the cell, the stream of nitrogen passed through the washing bottle filled with genuine seawater.

The amperometric titration of two defined concentrations of purified MT component ((0.78 and 1.55) × 10^{-8} mol/L) proceeded with addition of the standard $CdCl_2$ solution to seawater samples at two different pH's. After the titrant addition, the solution was equilibrated up to 30 s under stirring and in nitrogen purging conditions. Voltammetric measurements were performed in a DPASV mode.

Instruments and Apparatus

All amperometric measurements were performed with the polarograph PAR model 174A and a Hewlett-Packard recorder model 7004B. The potentiostatic control was achieved with the three-electrode system. The temperature was kept constant with the laboratory water circulator HAAKE D8–G. The pH was measured with an Orion Model EA 920 pH meter and a combined Ross Type 8102SC glass electrode.

RESULTS AND DISCUSSION

Titration of two defined concentrations of purified MT component (1.55 × × 10^{-8} and 0.78 × 10^{-8} mol/dm^3) proceeded with addition of the standard $CdCl_2$ solution to seawater samples at pH ≈ 1 and pH ≈ 8. The titration of 1.55 × 10^{-8} mol/dm^3 MT was performed in the range (0.089 to 9.79) × 10^{-7} mol/dm^3

$CdCl_2$, while the titration of 0.78×10^{-8} mol/dm^3 MT was performed varying the $CdCl_2$ concentration in the range (0.89 to 8.90) $\times 10^{-8}$ mol/dm^3. Voltammetric measurements in a differential pulse mode at pH $\approx$ 1 reveal the existence of only one signal of Cd(II), which corresponds to the Cd_{ionic} form, with the peak potential E_p = –0.52 V *vs.* the reference electrode, as indicated in Figure 1. This signal comprises oxidation of the deposited Cd-amalgam to both the hydrated Cd-cation and the Cd-chloride species. Voltammetric measurements of the solution of the same composition at pH $\approx$ 8 reveal, beside the signal of Cd_{ionic}, an additional signal of CdTh complex (where Th denotes the apoprotein molecule), with peak potential E_p = –0.68 V *vs.* the reference electrode (Figure 1). In a DPASV mode the half-width of Cd_{ionic} and CdTh

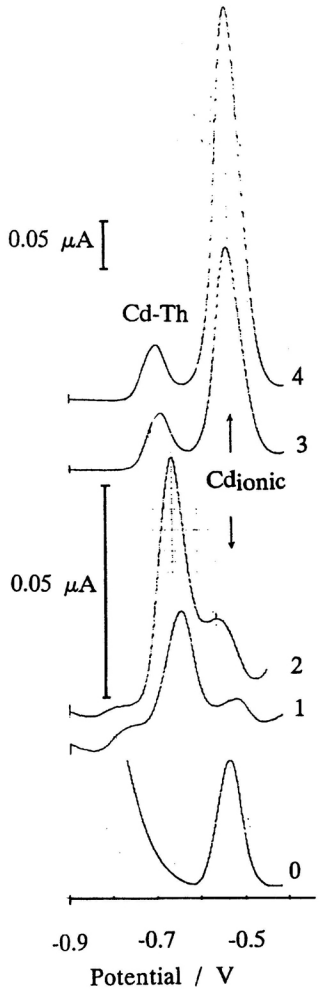

Figure 1. Current-potential curves of 1.55×10^{-8} mol/dm^3 MT recorded in a seawater sample of pH = 1.4 (curve 0) and pH = 8.3 (curve 1). Standard $CdCl_2$ solutions were added: 0.89×10^{-7} (curve 2); 4.45×10^{-7} (curve 3); 8.01×10^{-7} mol/dm^3 (curve 4). Two types of Cd(II) species recorded in a DPASV mode are assigned as CdTh and Cd_{ionic}. The sensitivity at which curves 0 to 2 were recorded is different from the one at which curves 3 and 4 were recorded.

signals amounts to 65 mV, which points to comparable reversibility of both electrode processes. Based on our previous studies in a DPASV mode, by which the formation of well defined Cd-NTA and Cd-EDTA complexes was followed, in the anodic stripping step the respective signal of Cd-complex species was not recorded due to the irreversible characteristics of the electrode process. Therefore, the fact that the CdTh signal is recorded in the anodic stripping step (Figure 1) supports the reversible character of this specific signal.

Based on the titration data of two different concentrations of MT component with $CdCl_2$, the amount of CdTh complex has been assessed:
A) indirectly, as the difference of Cd_{ionic} signal height in the presence of MT at pH $\approx$ 1 and pH $\approx$ 8;
B) directly, quantifying the signal height of the CdTh complex.

Procedure A) Titration data presented as found vs added Cd_{ionic} concentrations at 1.55×10^{-8} and 0.78×10^{-8} mol/dm^3 MT are shown in Figures 2 and 3, respectively. Each symbol on the graph represents a single measurement. The difference between the titration curves at pH $\approx$ 1 and pH $\approx$ 8 should indicate the complexation of Cd^{2+} by MT ligand. In the amperometric titration procedure, which refers to the complexing capacity determination,[13,14] only the peak height of Cd_{ionic} is evaluated. The peak height of Cd_{ionic} at pH $\approx$ 1 is assigned to the total cadmium concentration (Cd_T), while that one at pH $\approx$ 8 is assigned to the ionic cadmium concentration (Cd_i). The signal height of Cd_i has been calibrated according to the slope of the calibration straight-line for cadmium at pH $\approx$ 1. At two MT concentrations, the

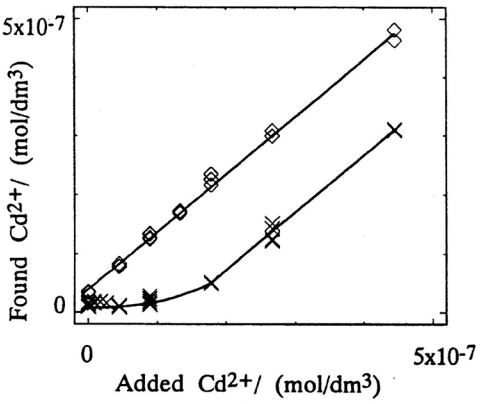

Figure 2. Amperometric titration data of 1.55×10^{-8} mol/dm^3 MT with the standard $CdCl_2$ solution in seawater samples at pH = 1.30 (◊) and pH = 8.11 (X), presented as found *vs.* added Cd-ionic concentrations.

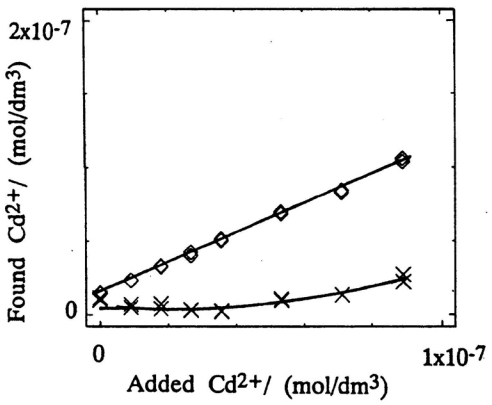

Figure 3. Amperometric titration data of 0.78×10^{-8} mol/dm^3 MT titrated with the standard $CdCl_2$ solution in seawater samples at pH = 1.76 (◊) and pH = 8.28 (X), presented as found *vs.* added Cd-ionic concentrations.

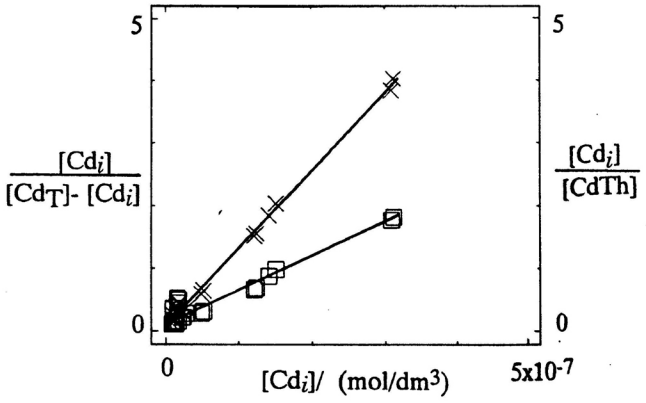

Figure 4. Amperometric titration data of overall 1.55×10^{-8} mol/dm^3 MT treated as: (□) the ratio ($[Cd_i] / [Cd_T] - [Cd_i]$) *vs.* $[Cd_i]$; (X) the ratio ($[Cd_i] / [CdTh]$) *vs.* $[Cd_i]$. The formation of the CdTh complex was directly estimated from the anodic signal at –0.68 V *vs.* the reference electrode.

data expressed as the ratio ($[Cd_i] / [Cd_T] - [Cd_i]$) *vs.* $[Cd_i]$ give the straight-lines of high correlation coefficients (Figures 4 and 5, symbol □). From the slope and the intercept, the ligand concentration C_L available for complexing Cd^{2+} ions and the apparent concentration stability constant K' have been determined, as summarized in Table I.

Procedure B) Titration data at 1.55×10^{-8} and 0.78×10^{-8} mol/dm^3 MT were additionally treated applying procedure B), so that the concentration

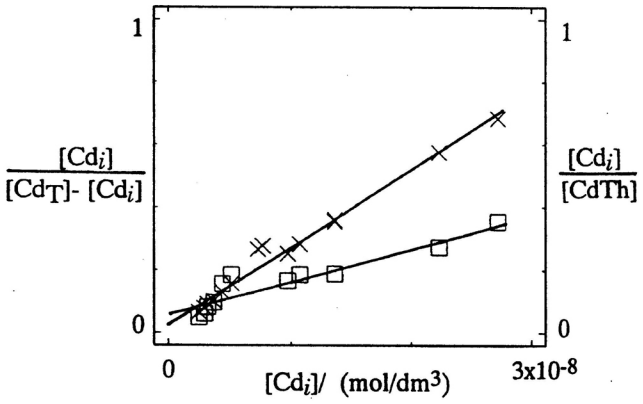

Figure 5. Amperometric titration data of overall 0.78×10^{-8} mol/dm^3 MT treated as: (□) the ratio ([Cd$_i$] / [Cd$_T$] – [Cd$_i$]) *vs.* [Cd$_i$]; (X) the ratio ([Cd$_i$] / [CdTh]) *vs.* [Cd$_i$]. The formation of the CdTh complex was directly estimated from the anodic signal at –0.68 V *vs.* the reference electrode.

of cadmium complexed by MT was directly assessed, quantifying the specific signal of the CdTh complex (Figure 1). The signal height of CdTh was calibrated according to the slope of the calibration straight line for cadmium at pH ≈ 1. At two MT concentrations, the data expressed as the ratio ([Cd$_i$] / / [CdTh]) *vs.* [Cd$_i$] give the straight-lines of high correlation coefficients (Figures 4 and 5, symbol *X*). From the slope and the intercept the ligand concentration C_L available for complexing Cd^{2+} ions and the apparent concentration stability constant K were determined. After a twofold increase of the ligand concentration ((0.78 to 1.55) $\times 10^{-8}$ mol/dm^3), the available ligand concentration C_L for complexing Cd^{2+} ions increased twice ((4.05 to 8.10) $\times 10^8$ mol/dm^3) (Table I).

TABLE I

The values of the ligand concentration C_L available for complexing Cd^{2+} ions and the apparent concentration stability constant K, determined by two evaluation procedures, A) and B). The data refer to seawater media, 0.7 mol/dm^3 ionic strength and 25 °C.

	Evaluation Procedure			
	A)		B)	
MT concentration mol/dm^3	C_L mol/dm^3	K (mol/dm^3)$^{-1}$	C_L mol/dm^3	K (mol/dm^3)$^{-1}$
1.55×10^{-8}	2.02×10^{-7}	2.46×10^7	8.10×10^{-8}	1.35×10^8
0.78×10^{-8}	9.58×10^{-8}	1.74×10^8	4.05×10^{-8}	9.56×10^8

The straight-line relationship (Figures 4 and 5) indicates that one type of functional groups on MT molecules exists and that the interaction of Cd^{2+} ions with MT molecule occurs via one type of functional groups. In agreement with the literature data[2,4,15] and our previous study,[16] Cd^{2+} is bound to the MT molecule via sulphydryl functional groups of cystein residues. A high correlation exists between the content of SH-groups and the Cd(II) content bound to these groups.[12]

If the available ligand concentration C_L determined by procedure A) or B) is normalized to the total MT concentration, the overall number of cadmium ions bound to the MT molecule could be defined. Applying the C_L values determined by procedure A), at two different MT concentrations, the molar ratio C_L/MT_t amounts to 12 and 13, respectively. Applying the C_L values determined by procedure B), at two different MT concentrations, the molar ratio C_L/MT_t amounts to 5.2. The molar ratio 5 is in agreement with the one previously determined from the concentration ratio of the CdTh complex and the total MT concentration.[17] The important conclusion has been drawn that irrespective of the MT concentration and the surplus of Cd^{2+} ions, overall 5 atoms of Cd^{2+} are bound per molecule of the *in vivo* induced mussel MT. For the mammalian type of MTs, whose metal-binding properties have been well characterized, the highest metal to ligand molar ratio has been defined as 7. Our data on the saturation of the *in vivo* induced mussel MT with Cd^{2+} ions indicate that, instead of 7, the highest molar ratio of mussel MT saturated with cadmium is 5. If the metal-binding stoichiometry of the mammalian and mussel types of MT is comparable, it would imply that the additional 2 binding positions on the MT molecule would remain unoccupied by cadmium. For the mammalian type of MT induced *in vivo* it has been reported that the cadmium/zinc stoichiometry of 5/2 has never been exceeded, the remaining 2 zinc atoms occupy a special position in the 3-metal cluster of the MT molecule.[18] We are not yet able to state that the additional 2 binding positions on the mussel MT molecule, induced *in vivo*, are occupied by zinc, but in the original, undiluted sample of purified MT component[12] the evaluated cadmium/zinc stoichiometry equals 3.7/2.5, which might suggest that, in the mussel type of MT, 2 zinc atoms occupy a specific position and could not be exchanged by Cd^{2+}.

Comparing the results of the titration data of two different MT concentrations, applying procedures A) and B), it can be concluded that if the data were treated according to procedure A), which is usually applied for complexing capacity determination of undefined ligands,[13] the higher available ligand concentration C_L, the lower apparent concentration stability constant K' and an unrealistically high C_L/MT_t ratio were determined. These facts suggest a certain inconsistency in the procedure of the complexing capacity determination. The reliability and applicability of the titration data treatment according to procedure A) could be at best evaluated with a well de-

fined type of ligand. The differences in the results of the two procedures, A) and B), are most probably caused by the unreliable estimation of the CdTh complex, indirectly determined as the difference ($[Cd_T] - [Cd_i]$). For the two evaluation procedures, the Cd_{ionic} concentrations are the same. The fact that the slope of the straight line ($[Cd_i] / [Cd_T] - [Cd_i]$) *vs.* $[Cd_i]$ is lower than that of ($[Cd_i] / [CdTh]$) *vs.* $[Cd_i]$, as noticeable from Figures 4 and 5 (symbols □ and X), respectively, indicates an erroneous estimation of the CdTh complex concentration by procedure A). It implies that the difference ($[Cd_T] - [Cd_i]$) and therefore the CdTh complex concentration are unrealistically high, which should be the subject of a further study. Therefore, the C_L values determined by procedure A) are higher than those determined by procedure B), as see from Table I. These results point out that the complexing properties of MTs could be reliably estimated only if the data of amperometric titration are based on the evaluation of the specific signal of the CdTh complex.

Applying the mass balance equations on cadmium and MT ligand, the apparent concentration stability constant K' has been defined:

$$K' = [CdTh] / ([Cd_i] (C_L - [CdTh])) . \quad (1)$$

From the straight-line (Figure 4 symbol X), in the range (0.1 to 4.0) × 10^{-7} mol/dm^3 Cd_{ionic}, the respective $[Cd_i] / [CdTh]$ ratio has been read off and inserted into Eq. (1). The concentration of MT not complexed with cadmium has been calculated as indicated in Eq. (1). The average K' for 15 data sets has been determined as (1.35 ± 0.01) × 10^8 (mol/dm^3)$^{-1}$, which is in a very good agreement with K' = 1.35 × 10^8 (mol/dm^3)$^{-1}$ determined from the slope and the intercept of the straight-line (Figure 4, symbol X). In the same manner, at 0.78 × 10^{-8} mol/dm^3 MT from the straight-line (Figure 5, symbol X) the average K' has been determined as (9.64 ± 0.05) × 10^8, which is in a very good agreement with K' = 9.56 × 10^8 (mol/dm^3)$^{-1}$ determined from the slope and the intercept of the straight-line (Figure 5, symbol X), according to procedure B).

The procedure of independent calculation of the stability constants confirms the fact that the K' is the apparent concentration stability constant of the CdTh complex because the concentration of cadmium is expressed in the ionic form and not as the hydrated $[Cd^{2+}]_{aq}$ one. The concentration stability constant K of CdTh complex has been determined taking into account the speciation of Cd_{ionic} in seawater,[19] *i.e.* the defined chloride-ion concentration and the stability constants of Cd-chloride complexes. At 0.7 mol/dm^3 ionic strength, the $[Cd^{2+}]_{aq}$ equals 2.6% of the Cd-ionic concentration, which is in agreement with our previous results on the speciation of cadmium in seawater.[19] At 0.7 mol/dm^3 and at 25 °C the corrected concentration stability constant of CdTh amounts to K = 5.18 × 10^9 and 3.72 × 10^{10} (mol/dm^3)$^{-1}$ at C_L = 8.10 × 10^{-8} and 4.05 × 10^{-8} mol/dm^3, respectively.

Acknowledgment. – The financial support of the Ministry of Science and Technology of the Republic Croatia is gratefully acknowledged.

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SAŽETAK

Elektrokemijsko određivanje svojstava metalotioneina izoliranih iz *M. galloprovincialis* za vezanje metala

Biserka Raspor i Jasenka Pavičić

Provedena je amperometrijska titracija određenih koncentracija metalotioneina standardnom otopinom $CdCl_2$ u morskoj vodi, pri pH ≈ 1 i pH ≈ 8, radi određivanja kompleksirajućih svojstava pročišćene komponente nmetalotioneina. Nastali kompleks kadmij-tionein određen je posrednim i neposrednim načinom. Uspoređeni su rezultati o raspoloživoj koncentraciji liganda C_L za kompleksno vezanje iona Cd^{2+} i prividnoj konstanti stabilnosti K', određeni na dva načina. Određivanje kompleksirajućeg svojstva metalotioneina pouzdano je ako se određivanje osniva na specifičnom signalu kompleksa Cd-tioneina.