

# Study of complexing properties of the $\alpha$ -metallothionein domain with cadmium and/or zinc, using differential pulse polarography

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## Abstract

A study of the complexing properties of the  $\alpha$ -metallothionein domain with cadmium and/or zinc was performed at pH 8, using differential pulse polarography. The applied method allows one to distinguish between the chemical form of the compound, free metal ions and complexes with the  $\alpha$ -domain as well as providing evidence that different forms of complexation exist. This technique was used to monitor changes of different species as a function of different parameters. Polarographic results have been compared with those obtained by electrospray ionisation mass spectrometry providing the molecular mass of compounds at different proportions of metal ions to the  $\alpha$ -domain, under similar experimental conditions. The  $\alpha$ -domain is capable of complexing cadmium and/or zinc, separately or after simultaneous additions of metal ions. In the case of binary mixtures, Cd +  $\alpha$  or Zn +  $\alpha$  pure complexes are formed, whereas in the case of tertiary mixtures  $\alpha$  + [Cd + Zn] mixed cadmium and zinc complexes prevail. When one of the two ions is added to a solution containing the  $\alpha$ -domain complexed with the other cation, it is incorporated into the molecule resulting in a reorganisation, which is illustrated by changes in the polarographic responses. Two different conditions can be clearly distinguished: an excess of ligand and when the metal ions are in excess. For these two conditions two different and characteristic features of polarograms were obtained. So, several stable forms of cadmium and zinc complexation with the  $\alpha$ -domain exist, depending on the composition of the solution.

The combination of results obtained using two different techniques was very useful for the assignment of different cadmium and zinc electrochemical responses. © 2000 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

The fundamental structure accepted for mammalian metallothioneins (MT) is the organisation of metal complexes in two different metal clusters. The  $\alpha$ -cluster corresponds to the carboxyl terminal domain containing eleven cysteinic residues and the  $\beta$ -cluster corresponds to the amino terminal domain

with nine cysteinic residues. The structure is controlled by the formation of metal-thiolate bonds in which all 20 cysteines participate. The characteristics and properties of MT have been studied using a great variety of analytical methods and described in different monographs [1–7]. However, there have been relatively few studies with the  $\alpha$ - and  $\beta$ -domains separately probably due to the difficulties associated with their separation and purification. In fact, these two isolated domains can be obtained by the solid-phase and fragment condensation methods or isolated after proteolytic digestion of MT of different origin.

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Nevertheless detailed knowledge of the behaviour of well-characterised isolated  $\alpha$ - and  $\beta$ -domain and particularly of their complexation properties with metal ions should contribute significantly to a better understanding of the entire MT.

The aim of this research is the systematic study of complexing properties of the  $\alpha$ -MT domain with cadmium and/or zinc using electroanalytical methods. These methods, particularly differential pulse polarography (DPP) have been widely applied by our team to the characterisation of Cd, Zn MT [8–17] and related molecules such as the peptidic fragment Lys-Cys-Thr-Cys-Cys-Ala (56–61) MT 1 (FT) being part of the MT structure, alone and in the presence of cadmium and/or zinc [19–26]. These studies have been very useful for the identification and assignment of voltammetric peaks; in fact, in the case of Cd, Zn MT and of Cd, Zn and FT mixtures at least three well defined responses were obtained using the mercury electrode, one of them is due to the amino acid part of the molecule because of the electroactivity of thiol groups and the two other peaks due to the reduction of cadmium and zinc which is electrochemically reversible at the mercury electrode. In addition the electrochemical response peak potential and peak intensity allows the distinction of the chemical forms of the element between free and complexed metal ions and also allows one, on the one hand, to identify different species and, on the other hand, to follow the evolution of these species as a function of different parameters.

The obtention of the  $\alpha$ -domain and the investigation of its characteristics have been reported [3,7,27–31]. The synthesised peptide used in this work has been purchased from the Biochemistry Institute of Zurich University. The purity of this molecule was checked by both, Dr. Klauser's team from the Zurich University and our team, using electrospray ionisation mass spectrometry (ESI-MS) [32,33].

Different types of additions of metal ions to the  $\alpha$ -domain and the inverted additions of the  $\alpha$ -domain to metal ions have been performed and changes in the composition of the dissolution have been monitored using DPP with the aim of investigating the binding and metal exchange properties. Firstly, binary mixtures, obtained by the gradual addition of metal ions separately has been studied and secondly tertiary mixtures of  $\alpha$ , cadmium and zinc obtained by gradual addition of cadmium and zinc, either

simultaneously or sequentially, have been investigated. The use of  $\alpha$ -MT domain has the advantage over the Cd, Zn MT that it permits one to study the behaviour of the cadmium and zinc complexes in the  $\alpha$ -cluster separately as well as the influence of one metal ion on the complexing properties of the other.

The polarographic results with the  $\alpha$ -domain are compared with those obtained with the  $\beta$ -domain under identical experimental conditions [34] and also with those obtained recently by our team [33] on different mixtures  $\alpha$ , cadmium and/or zinc using ESI-MS.

## 2. Experimental

### 2.1. Apparatus

All measurements were performed using a potentiostat BAS Model 100B/W electrochemical analyser, connected to a BAS Controlled Growth Mercury Electrode polarographic stand equipped with a HDME as the working electrode, a Ag/AgCl reference electrode and a platinum wire as an auxiliary electrode. This equipment was introduced into a glove box, PLAS LABS, Inc. 830-ABC Series Chambers, connected to a diaphragm pump VACUUBRAND GMBH + CO model ME-4, so as to avoid oxidation of the MT thiol groups by the O<sub>2</sub> present in the air during sample manipulation. The purging gas used was 99.999% N<sub>2</sub>.

A pH meter, Orion Model 920A, equipped with a glass electrode combined with an Ag/AgCl reference electrode, was employed for the pH measurements.

### 2.2. Chemicals

$\alpha$ -domain sample, of which the amino acidic sequence corresponds to a human MT, was synthesised and purified by the Biochemistry Institute of Zurich University, by peptide synthesis, following the procedure described in [32].

### 2.3. HuMT- $\alpha$ (31 residues)

H-Lys-Ser-Cys-Cys-Ser-Cys-Cys-Pro-Val-Gly-Ala-Lys-Cys-Ala-Gln-Gly-Cys-Ile-Cys-Lys-Gly-Ala-Ser-Asp-Lys-Cys-Ser-Cys-Cys-Ala-OH.

No impurities or metal cations are detected.  $\alpha$ -domain was provided in powder form, and stored

at  $-30^{\circ}\text{C}$  without any further purification. Samples were freshly prepared before measurements under an inert atmosphere.

The supporting electrolyte was prepared from equimolar amounts of phosphoric acid and 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulphonic acid (HEPES) and was used as a buffer, adjusting to pH 8 by addition of NaOH.

Cadmium and zinc solutions used in the study of metal additions were prepared from Tritisol standard solutions of  $1000\text{ mg l}^{-1}$  purchased from Merck.

All solutions were prepared using deionised water obtained from an ultra-pure water system, MilliQ, plus Model 185 (Millipore SA, Molsheim, France), and deoxygenated before use, by purging with  $\text{N}_2$ . All the reagents employed were of *analytical grade*.

Hexadistilled mercury, grade 9N, used by the polarographic stand was purchased from Rhone-Alpes Mercure. Purging was carried out with 99.999%  $\text{N}_2$ .

#### 2.4. Electrochemical procedure

The supporting electrolyte was purged with nitrogen for 10 min before the start of the experiments. The samples were purged continuously between each measurement and, during the experiment, nitrogen was passed over the cell. All of the studies were carried out in an inert atmosphere inside a glove box at room temperature.

Each sample was measured by DPP over a large cathodic scan range of potentials, from  $-200$  to  $-1350\text{ mV}$ , with the aim of obtaining a good development of the voltammetric peaks.

Experimental parameters used had been optimised in previous works [11]. In all cases, the drop size is the same and depends on the number of pulses ( $n_p = 5$ ) and the pulse width ( $p_w = 10\text{ ms}$ ). DPP was carried out using a scan rate of  $4\text{ mV s}^{-1}$  with a drop time of 1 s and the pulse amplitude fixed at  $10\text{ mV}$ .

All peak potential and peak current data presented in this paper were obtained by means of PeakFit V.4.1, a calculation programme from Jandel Scientific Software for Windows. After importing the files containing the raw data, the polarograms were smoothed using a Loes algorithm, followed by a manual baseline subtraction choosing a *linear* option from the 2nd Deriv. Zero algorithm. The *AutoFit Peaks I residuals* option, which identifies peaks by identifying local maxima in

a smoothed data stream, was employed to measure the polarographic peaks, minimisation being performed by Least Squares. The *asymmetric logistic peak* function produced the best results for the adjustment of peaks generated to the original polarograms:

$$y = a_0 \left[ 1 + \exp \left( -\frac{x + a_2 \ln a_3 - a_1}{a_2} \right) \right]^{-a_3 - 1} a_3^{-a_3} \\ \times (a_3 + 1)^{a_3 + 1} \exp \left( -\frac{x + a_2 \ln a_3 - a_1}{a_2} \right)$$

where  $a_0$  is the amplitude,  $a_1$  the centre,  $a_2$  the width and  $a_3$  is the shape of the peak.

The aim of this experiment is to monitor the evolution of a solution containing either the  $\alpha$ -domain or one of the metal ions selected for this work, Cd or Zn, when gradual additions of the rest of the components are performed following a pre-established order. The experiments programmed are

- addition of Cd to the  $\alpha$ -domain, and subsequent addition of Zn;
- addition of Zn to the  $\alpha$ -domain, and subsequent addition of Cd;
- addition of the  $\alpha$ -domain to Cd, and subsequent addition of Zn;
- addition of the  $\alpha$ -domain to Zn, and subsequent addition of Cd;
- addition of an equimolar solution of Cd and Zn to the  $\alpha$ -domain;
- addition of the  $\alpha$ -domain to an equimolar solution of Cd and Zn.

### 3. Results and discussion

#### 3.1. Polarographic behaviour of the $\alpha$ -MT domain during sequential additions of cadmium and zinc, separately

Sequential additions of cadmium and zinc separately to a solution containing the  $\alpha$ -domain have been carried out at pH 8, at which the complete formation of  $\alpha$ -complexes with Cd and Zn is reached. The inverted additions of the  $\alpha$ -domain to separate solutions of cadmium and zinc have also been performed at the same pH. With the aim of identifying the polarographic peaks, various selected polarogrammes are shown in Fig. 1, corresponding to the additions of

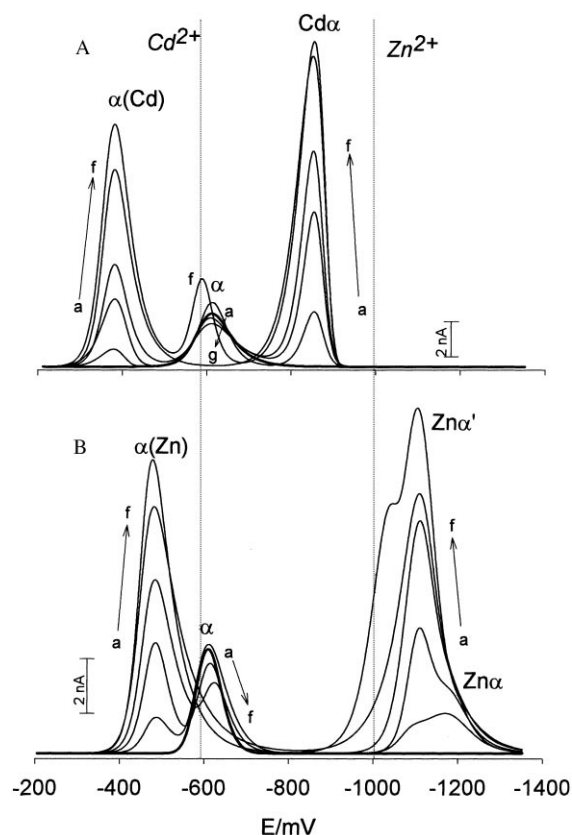


Fig. 1. DPP polarograms corresponding to the sequential addition of Cd or Zn (mol/mol $\alpha$ ) to a  $\alpha$ -MT  $5.5 \times 10^{-6}$  M in HEPES-phosphate buffer  $10^{-2}$  M at pH 8.0. (A) Additions of Cd: (a) 0.0; (b) 0.3; (c) 0.9; (d) 1.4; (e) 2.7; (f) 3.6; and (B) additions of Zn (a) 0.0; (b) 0.3; (c) 0.9; (d) 1.8; (e) 2.7 and (f) 3.6.

cadmium (Fig. 1a) and zinc (Fig. 1b) to the  $\alpha$ -domain. In Fig. 1 the potentials corresponding to free cations,  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$ , in the same electrolyte support, are indicated by vertical lines. The polarographic peaks have been assigned in agreement with our own results obtained in previous works with Cd, Zn MT of different origin [8–17] and with mixtures of FT, cadmium and/or zinc [18–26] using DPP, square wave voltammetry (SWV) and cyclic voltammetry (CV). In each of the initial polarogrammes (Fig. 1A and B, curve a) the  $\alpha$ -peak due to the  $\alpha$ -domain alone is observed; this  $\alpha$ -peak is better defined than that corresponding to the  $\beta$ -domain previously described [34]. The  $\alpha$ -peak potential has the same value using both DPP and SWV techniques; however, using the latter

technique the  $\alpha$ -peak is very well defined exhibiting a high intensity due to the adsorption phenomenon. The  $\alpha$ -peak is attributed to the oxidation of the mercury electrode in the presence of free thiol groups of the molecule not complexed by the metal ions giving rise to the mercury thiolate complex, probably adsorbed at the electrode surface. Peaks labelled  $\alpha(\text{Cd})$  and  $\alpha(\text{Zn})$  are due to the oxidation of the mercury electrode in the presence of thiol groups complexed with cadmium and zinc, respectively.  $\text{Cd}\alpha$ ,  $\text{Zn}\alpha$  and  $\text{Zn}\alpha'$  correspond to the reduction of metal ions complexed with the  $\alpha$ -domain in given complexation forms. The electrochemical reactions describing the oxidation and reduction processes are reported elsewhere [8,14].

It is observed in Fig. 1 that the reduction potentials of free and complexed cadmium are well defined and separated and hence the different chemical forms of this element can be clearly distinguished. On the contrary, several polarographic signals with very close peak potentials are obtained for the reduction of free and complexed zinc and, consequently in this case, the attribution of different responses to several chemical forms of zinc is arduous. For this reason, a special software was applied in order to improve the resolution of all polarographic peaks, particularly those corresponding to Zn(II) reduction. Throughout this paper all values of peak potentials and peak current are those obtained with the calculation programme PeakFit V.4.1, as is mentioned in the Section 2. However, it is necessary to point out that whereas in the case of cadmium both, electrochemical and mathematical approaches, the peak potentials are identical, in the case of zinc reduction, for which three different peaks are detected, the experimental potential value and that obtained after treatment could be slightly different. It is illustrated by the difference of potential for the same peak ( $\sim 30$  mV) obtained from one experiment to another. Nevertheless the application of a mathematical program is the only solution at present for resolving the Zn(II) peaks.

Fig. 2 shows the variation of peak current and hence the evolution of species in dissolution as a function of the added compounds, i.e. the metal ions (Fig. 2a and b) and the  $\alpha$ -domain (Fig. 2c and d). Standard additions have been carried out until either the appearance of a signal corresponding to free cations or the disappearance of this response, which is easy to detect in the case of cadmium but not in the case of zinc.

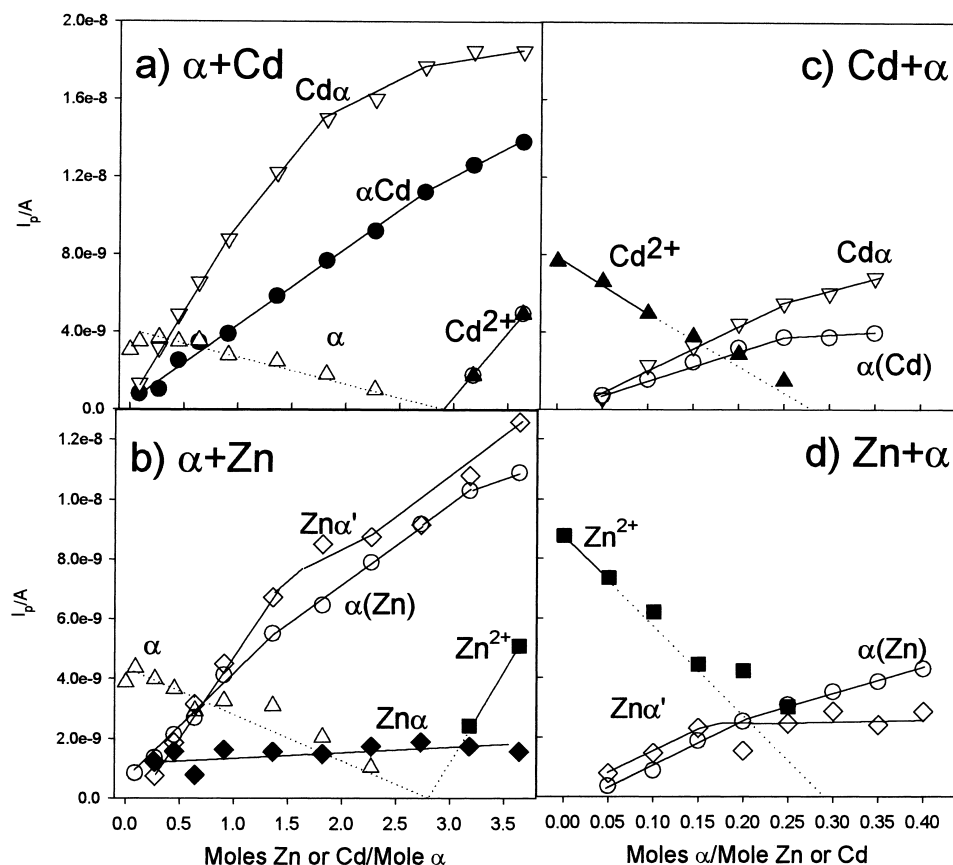


Fig. 2. Peak current evolution vs. the amount of Cd (a) or Zn (b) added to a  $\alpha$ -MT  $5.5 \times 10^{-6}$  M in HEPES-phosphate buffer  $10^{-2}$  M at pH 8.0; and vs. the amount of  $\alpha$  added to both solutions  $5 \times 10^{-6}$  M of Cd (c) and Zn (d) in the same media.

In spite of the bad definition of the  $\alpha$ -peak, it is observed that the signal corresponding to the free  $\alpha$ -domain disappears at the proportion of added cation  $[M]_{ad} \geq 3 \text{ mol M/mol } \alpha$ . The detection of free  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$  starts to be observed for this same proportion. In the case of  $\alpha$ -domain additions to metal ions, the corresponding responses of free  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$  disappear at the proportion of  $[\alpha]_{ad} \geq 0.25\text{--}0.3 \text{ mol } \alpha/\text{mol M}$ . It is necessary to point out that the peak potential due to  $\text{Zn}^{2+}$  shifts to more negative potential for the third addition of  $\alpha$  ( $\geq 0.1 \text{ mol } \alpha/\text{mol Zn}$ ); this behaviour has already been observed during studies of standard addition of metal ions to MT [13–16] and to mixtures of FT with cadmium and/or zinc [24,25]. The polarographic peaks due to the oxidation,  $\alpha(\text{Cd})$  and  $\alpha(\text{Zn})$  and the reduction,  $\text{Cd}\alpha$ ,  $\text{Zn}\alpha'$  and  $\text{Zn}\alpha$ , of the metal complexes

gradually increase until they reach an unchanged value. These plateaux are better defined for  $\alpha(\text{Cd})$  and  $\text{Zn}\alpha'$  peaks in the case of  $\alpha$  additions to the cations (Fig. 2c and d) that in the case of inverted additions (Fig. 2a and b).

Moreover  $\alpha$ -peak is not detected when this compound is added in excess,  $[\alpha]_{ad} > 0.3$  (Fig. 2c and d) although it is detected during the inverted additions (Fig. 2a and b). Likewise the peak current for the reduction of complexed Zn(II) is smaller than that of the reduction of free  $\text{Zn}^{2+}$  in the initial solution ( $I_{p_{\text{Zn}^{2+}}} \approx 4I_{p_{\text{Zn}\alpha'}}$ ) (Fig. 2c) indicating either that the access to the electrode surface seems to depend on the position occupied by the Zn(II) in the complexed molecule and it is less easy than in the case of free  $\text{Zn}^{2+}$  or that a change in the reversibility of the Zn(II) system could occur. This difference in the peak current has already

been observed in the study of the complexation of zinc with the  $\beta$ -domain of MT [34].

What can be derived from this data is that the addition of Cd or Zn to the  $\alpha$ -domain or the inverted additions produce the complete complexation in a proportion  $M:\alpha \approx 3\text{--}4:1$ , which is in full agreement with the structure generally accepted for the  $\alpha$ -cluster of MT, which is able to bind four bivalent metal ions through its eleven cysteine residues.

In the case of  $Cd\alpha$  complex(es) the only peak detected for the reduction of Cd(II) is  $Cd\alpha$  which corresponds to the only complexation form of cadmium in binary mixtures  $\alpha + Cd$  (Fig. 2a and c). In the case of zinc, when this cation is added to an excess of  $\alpha$ -domain, both  $Zn\alpha$  and  $Zn\alpha'$  are simultaneously present (Fig. 2b), but not in the case of inverted additions,  $\alpha$  to excess of Zn, for which the only complexation form corresponds to that named  $Zn\alpha'$  (Fig. 2d). In both cases the prevalent form is  $Zn\alpha'$ , for which the intensity increases similarly to that of  $\alpha(Zn)$ , in agreement with the proposed electrochemical reactions, because the same compounds, i.e. the complex  $Zn\alpha$  is directly involved in the oxidation and reduction reactions. However, for  $\beta + Cd$  additions (Fig. 2a) at  $[Cd]_{ad} < 2$ ,  $Cd\alpha$  peak current linearly increases with a slope double that of the  $\alpha(Cd)$  peak in a similar manner to the behaviour of  $\alpha + Cd$  binary mixtures. This is difficult to explain if the electrochemical mechanism previously proposed is assumed; in fact, for the oxidation process, the Hg(II) coming from the electrode has to occupy the same complexation positions as those initially occupied by the cadmium. In previous studies performed with the peptidic fragment FT in the presence of cadmium at a proportion  $FT: Cd = 1:1$  the similarity of slopes if  $I_{pFT(Cd)}$  and  $I_{pCdFT}$  versus the total concentration has been verified [23].

### 3.2. Polarographic behaviour of the $\alpha$ -MT domain during sequential additions of cadmium and zinc simultaneously

With the aim of obtaining more information about complexing properties of the  $\alpha$ -domain with both cations, cadmium and zinc, are simultaneously present, the gradual additions of an equimolar mixture of Cd and Zn to the  $\alpha$ -domain and the inverted additions of  $\alpha$ -domain to an equimolar mixture of cadmium and zinc were performed. These additions

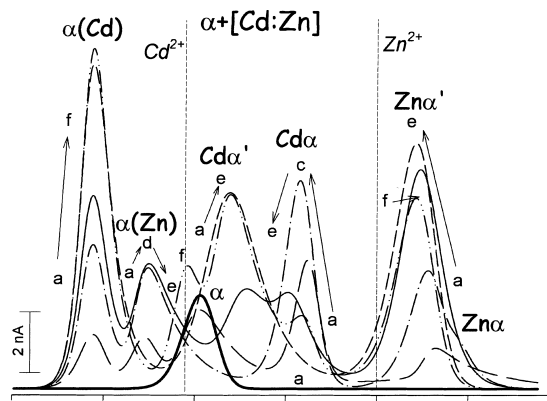


Fig. 3. Polarograms obtained by sequential additions of an equimolar solution of Cd and Zn to  $\alpha$ -MT  $5.5 \times 10^{-6}$  M in HEPES-phosphate buffer  $10^{-2}$  M at pH 8.0 (a) 0.0 mol Cd or Zn/mol  $\alpha$ ; (b) 0.8; (c) 2.0; (d) 2.8; (e) 7.2 and (f) 11.4.

were carried out until an excess of the added compounds was obtained. An example of several significant polarogrammes is depicted in Fig. 3. In Fig. 3 the peak labelled  $Cd\alpha'$  can be seen; this peak is attributed to the reduction of Cd(II) contained in the  $\alpha$ -cluster in a different complexation form to the  $Cd\alpha$  one, by analogy with peaks labelled  $CdT'$ ,  $CdFT'$  and  $Cd\beta'$  identified in previous works [9–26,34]. This peak is detected and sometimes becomes prevalent under given experimental conditions, particularly in the presence of zinc.

The evolution of the peak intensities of all species as a function of the concentration of the added compound is represented in Fig. 4, for the additions of cations to the  $\alpha$ -domain or for the inverted additions. In Fig. 4 the evolution of peak intensities corresponding to binary mixtures (see Fig. 2) is also represented with the aim of comparing the behaviour of binary and tertiary mixtures. Vertical lines indicate the proportion either of cations or of the  $\alpha$ -domain at which complete complexation is reached. In this Figure, different proportions  $\alpha:M$  can be distinguished at which different species can be detected or prevalent. At (1)  $[M]_{ad} < 2$  corresponding to an excess of  $\alpha$ , the  $\alpha$ -response corresponding to the  $\alpha$ -domain electrochemical reactions is very well detected with a polarographic peak well defined (Fig. 3a). The peak intensity decreases until its complete disappearance at  $[M]_{ad} > 4$  (Fig. 4a). Peaks corresponding to the oxidation of the mercury electrode,  $\alpha(Cd)$  and  $\alpha(Zn)$ , increase with very similar

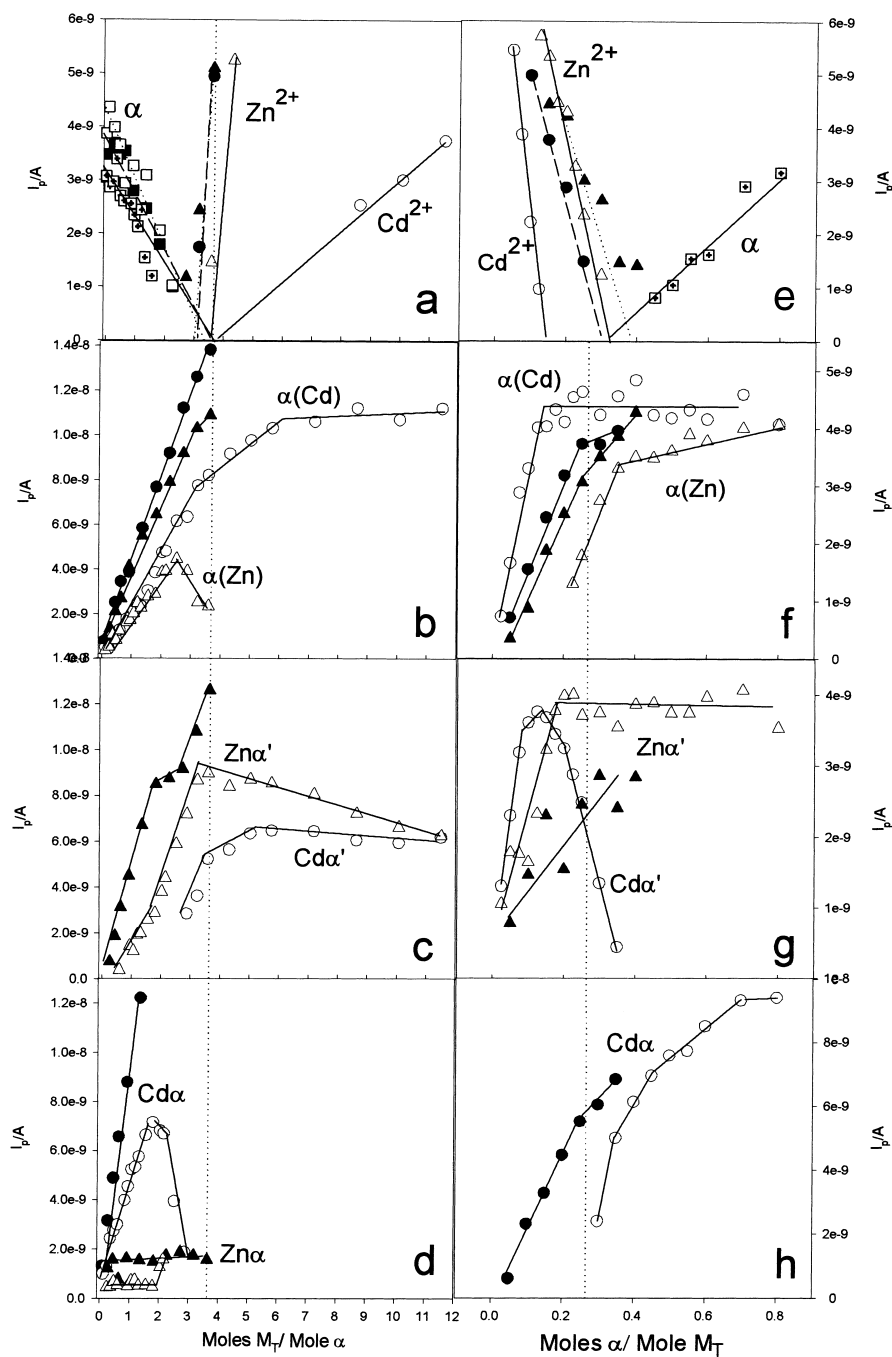


Fig. 4. Peak currents obtained for all peaks when: Cd (●), Zn (▲), or an equimolar solution of Cd and Zn (○) for Cd(II) peaks and (△) for Zn(II) peaks) were added to a solution of  $\alpha$ -MT  $5.5 \times 10^{-6}$  M (a–d); and when  $\alpha$ -MT is added to Cd (●), Zn (▲), or an equimolar solution of Cd and Zn (○) for Cd(II) peaks, and (△) for Zn(II) peaks), being the initial concentration of all cations in solution  $5 \times 10^{-6}$  M (e–h). Electrolyte: HEPES-phosphate buffer  $10^{-2}$  M at pH 8.0.

values (Fig. 4b).  $I_{p_{Cd\alpha}}$  grows with a slope value twice the  $I_{p_{\alpha(Cd)}}$  one, like in the case of binary mixtures (see Fig. 2a) and reaches a maximum value at  $[M]_{ad} \approx 2$  corresponding to a proportion  $\alpha: Cd: Zn \approx 1:1:1$  and then sharply decreases until its disappearance at  $[M]_{ad} > 3$  (Fig. 4d). The prevalent peak of Zn(II) reduction is  $Zn\alpha'$  which increases with a similar slope to the  $(\alpha)Cd$  and  $(\alpha)Zn$  peaks (Fig. 4c). (2)  $2 < [M]_{ad} < 4$ ,  $Cd(\alpha)$  increases with the same slope as in (1),  $\alpha(Zn)$  decreases and it is not observed at  $[M]_{ad} > 4$  (Fig. 4b).  $Cd\alpha'$ , the only peak due to the reduction of Cd(II), increases with a similar slope to the  $Zn\alpha'$  one and reaches an unchanged intensity at  $[M]_{ad} \geq 5$ .  $\alpha$ -peak disappears at  $[M]_{ad} \approx 4$  as mentioned above indicating the complete complexation of  $\alpha$  with metal ions (Fig. 4a). (3)  $[M]_{ad} > 4$ .  $Cd^{2+}$  and  $Zn^{2+}$  peaks corresponding to free cadmium and zinc start to be detected at  $[M]_{ad} > 4$ . In an excess of cations the following peaks are detected:  $\alpha(Cd)$ ,  $Cd\alpha'$  and  $Zn\alpha'$ , with intensities almost unchanged.

During the sequential additions of  $\alpha$ -domain to an equimolar mixture of both cations, several relations  $\alpha$  to  $M$  can also be distinguished to assess the predominance of different species (1)  $[\alpha]_{ad} < 0.15$ , the  $Cd^{2+}$  peak disappears at  $[\alpha]_{ad} \approx 0.15$  mol/mol MT corresponding to a proportion  $\alpha: Cd: Zn \approx 1:3:3$ . The disappearance of free cadmium before free zinc (Fig. 4e) is in complete agreement with the values of apparent complexation constants of  $\alpha$  with cadmium and zinc,  $K'_{Cd\alpha} > K'_{Zn\alpha}$  (vide infra). At this proportion, both  $\alpha(Cd)$  the only anodic peak corresponding to the oxidation of mercury and  $Cd\alpha'$ , the only peak due to the reduction of Cd(II), increase in intensity with a similar slope until reaching a maximum (Fig. 4f and g); then at  $[\alpha]_{ad} > 0.15$ ,  $\alpha(Cd)$  current remains unchanged and  $Cd\alpha'$  peak intensity decreases and is not observed at  $[\alpha]_{ad} > 0.35$ .  $Zn\alpha'$  peak increases, reaches a maximum at  $[\alpha]_{ad} \approx 0.2$  and then remains constant (Fig. 4g). (2)  $0.15 < [\alpha]_{ad} < 0.35-0.4$ . The free  $Zn^{2+}$  peak disappears at  $[\alpha]_{ad} \approx 0.25$  corresponding to a proportion  $\alpha: Cd: Zn$ , 1:2:2; at higher proportions free  $\alpha$  is detected (Fig. 4e). The  $Cd\alpha$  peak starts to be detected at  $[\alpha]_{ad} > 0.3$  which coincides with the disappearance of  $Cd\alpha'$ , indicating that, with an excess of ligand, the only complexation form of cadmium is the  $Cd\alpha$  one (Fig. 4h).  $\alpha(Zn)$  is detected at  $[\alpha]_{ad} > 0.2$ , increases up to  $[\alpha]_{ad} \approx 0.35$  and then remains constant (Fig. 4f). The polarogram's features are unchanged at

$[\alpha]_{ad} > 0.7$  exhibiting  $\alpha(Cd)$ ,  $\alpha(Zn)$ ,  $Cd\alpha$  and  $Zn\alpha'$  peaks corresponding to an excess of the  $\alpha$ -domain.

The detection of different peaks and hence the predominance of different species depend on the relative concentration between the  $\alpha$ -domain and the metal ions and also on the simultaneous presence of both cations in the tertiary mixtures or if the cations are added alone in the case of binary mixtures. The disappearance of free cations (Fig. 4e) and of free  $\alpha$  (Fig. 4a) coincides at the same proportion between total cations and the  $\alpha$ -domain,  $\alpha: M = 1:4$  in agreement with results of  $\alpha$ -domain studies previously published [3,7]. This proportion is the same for binary mixtures and when metal ions are present together (Fig. 4e) the response due to free  $Cd^{2+}$  disappears at  $\alpha: Cd: Zn$  1:3:3, at which the  $Zn^{2+}$  peak is yet detected, indicating the higher stability of the  $Cd\alpha$  complex. Concerning anodic responses, differences between binary and tertiary mixtures exist; so, in excess of  $\alpha$ -domain,  $\alpha/M > 0.25$ ,  $\alpha(Cd)$  and  $\alpha(Zn)$  behave in a similar manner for binary and tertiary mixtures (Fig. 4b and f); however  $\alpha(Zn)$  is not detected when the cations are in excess  $\alpha/M < 0.25$  and the only anodic peak corresponds to  $\alpha(Cd)$ . This seems to indicate that cadmium and zinc are occupying different positions in the molecule and zinc can be replaced by Hg(II) only in some of them. Likewise, the  $Cd\alpha'$  peak which is observed in the presence of zinc (Fig. 4c and g) is the prevalent one in an excess of cations, while the  $Cd\alpha$  peak is the only one due to the reduction of Cd(II) in the binary mixtures and in an excess of  $\alpha$ -domain (Fig. 4d and h).  $Cd\alpha'$  and  $Zn\alpha'$  are interrelated and are the prevalent peaks of the metal ions reduction when they are in excess in relation to  $\alpha$  (Fig. 4c and g). Both electrochemical responses are probably due to the same compound, a mixed complex of cadmium and zinc with  $\alpha$  (vide infra).

According to our proposed electrochemical reactions [8,14] the intensities due to, on the one hand,  $\alpha(Cd)$  and  $Cd\alpha$  or  $Cd\alpha'$  systems and, on the other hand, to  $\alpha(Zn)$  and  $Zn\alpha$  or  $Zn\alpha'$  systems should be similar because the same compounds, the complexes  $Cd\alpha$  and  $Zn\alpha$  are involved in the oxidation and reduction reactions; however  $I_{p_{\alpha(Cd)}}$  in an excess of cations (Fig. 4b) has the same value as the addition of the intensities of the cathodic peaks  $I_{p_{Cd\alpha}} + I_{p_{Zn\alpha}}$  (Fig. 4c) which can be explained if it is assumed that cadmium and zinc are occupying different complexation positions, for which the reduction of metal ions is possible



(Fig. 4c) but the oxidation of the mercury electrodes in the presence of thiol groups saturated with cadmium and zinc is only possible in the form corresponding to the  $\alpha(\text{Cd})$  reaction (Fig. 4b). The same behaviour is observed when the  $\alpha$ -domain is in excess; so  $I_{p_{\alpha}(\text{Zn})}$  (Fig. 4f) has an analogous value to  $I_{p_{\text{Zn}\alpha'}}$  (Fig. 4g) whereas  $I_{p_{\text{Cd}\alpha}}$  (Fig. 4h) has twice the value of  $I_{p_{\alpha}(\text{Cd})}$  (Fig. 4f), a similar ratio to that found during the study of binary mixtures,  $\alpha + \text{Cd}$  in an excess of  $\alpha$  (Fig. 2a).

Summarising all results (Fig. 4) it can be concluded that in an excess of  $\alpha$  and in the simultaneous presence of both cations the polarographic response is  $\alpha(\text{Cd})$ ,  $\text{Cd}\alpha$  and  $\text{Zn}\alpha$  or  $\text{Zn}\alpha'$  probably corresponding to the pure complexation forms of  $\alpha$  with cadmium and zinc and, in an excess of metal ions, when the limiting concentration is the  $\alpha$  one, a reorganisation of the form of complexation into the cluster is produced, probably leading to the form of cadmium and zinc mixed complexes; in this case the polarographic response is  $\alpha(\text{Cd})$ ,  $\text{Cd}\alpha'$  and  $\text{Zn}\alpha'$ . This transition of one form into another form implying the reorganisation of the molecule is clearly illustrated in Fig. 5 in which the total intensities of  $\text{Cd}(\text{II})$ , i.e.  $I_{p_{\text{Cd}^{2+}}} + I_{p_{\text{Cd}\alpha'}} + I_{p_{\text{Cd}\alpha}}$ , and  $\text{Zn}(\text{II})$ , i.e.  $I_{p_{\text{Zn}^{2+}}} + I_{p_{\text{Zn}\alpha'}}$ , reduction is represented as a function of the proportion of  $\alpha$  added for a given total concentration of cadmium and zinc equal to the initial one. It can be seen in Fig. 5 that whereas the total intensity due to the  $\text{Zn}(\text{II})$  remains constant

until  $[\alpha]_{\text{ad}} \geq 0.25$  corresponding to  $\alpha:\text{M} = 1:4$ , the response of complexed zinc is the half of free zinc,  $I_{p_{\text{Zn}\alpha'}} \approx 1/2 I_{p_{\text{Zn}^{2+}}}$ . On the contrary, the total response of  $\text{Cd}(\text{II})$  decreases until  $[\alpha] < 0.25$  at which point the  $\text{Cd}(\text{II})$  is complexed in the  $\text{Cd}\alpha'$  form, at  $[\alpha] > 0.25$  the complete transformation of the  $\text{Cd}\alpha'$  species into the  $\text{Cd}\alpha$  one occurs and, in the latter form, the complexed  $\text{Cd}(\text{II})$  gives a similar response to the free  $\text{Cd}^{2+}$ . It is necessary to point out that the complete complexation of the  $\alpha$ -domain with metal ions coincides with the minimum intensity of the  $\text{Cd}(\text{II})$  reduction, indicating an access to the electrode which is probably impeded by the structure of the molecule itself with all thiol groups binding cadmium and zinc simultaneously. In addition, the maximum intensity values for  $\text{Cd}\alpha'$  and  $\text{Zn}\alpha'$  peaks are the same, supporting the hypothesis that both responses come from the same compound. Once more the different behaviour of complexes  $\text{Cd}\alpha$  and  $\text{Zn}\alpha$  is revealed.

### 3.3. Polarographic behaviour of the $\alpha$ -MT domain during sequential additions of Cd or Zn followed by additions of Zn or Cd

After having added one of the metal ions to the  $\alpha$ -domain and the inverted additions (vide supra Figs. 1 and 2), the addition of the other cation was carried out with the aim of better understanding the

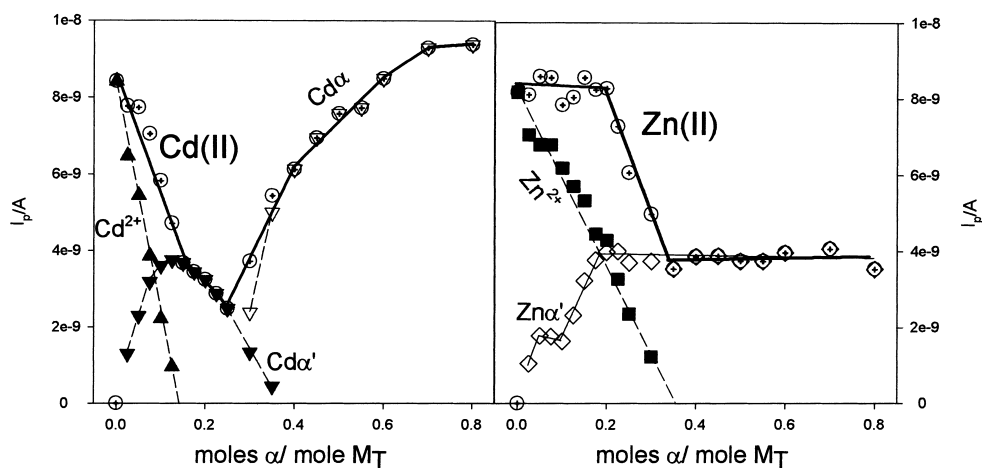


Fig. 5. Peak current measured for the reduction peaks produced when  $\alpha$ -domain is added to a solution containing Cd (right) or Zn (left)  $5 \times 10^{-6}$  M in HEPES-phosphate buffer  $10^{-2}$  M at pH 8.0. The total current corresponding to the sum of the reduction peaks for either  $\text{Cd}(\text{II})$  or  $\text{Zn}(\text{II})$  are represented in the figure by the symbol  $\odot$ .

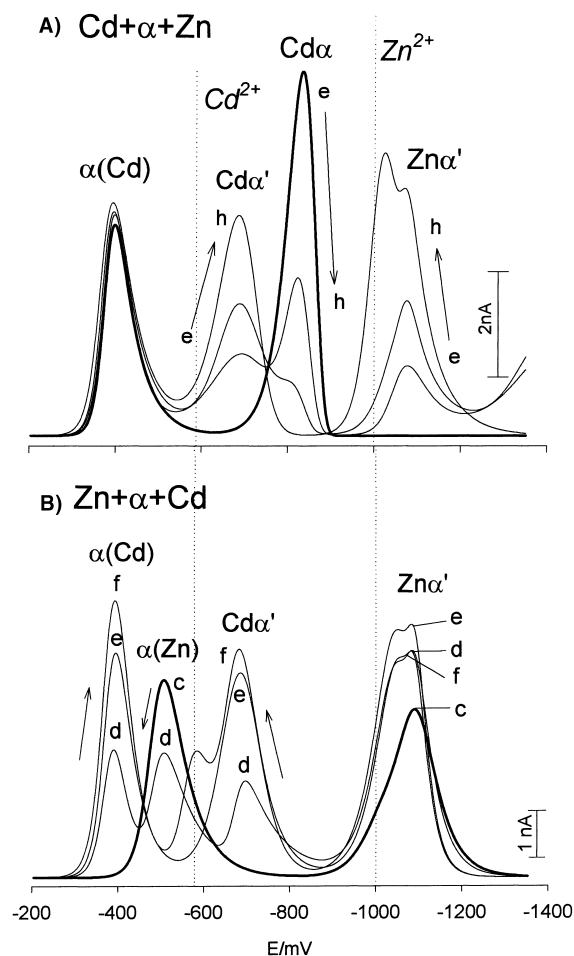


Fig. 6. Polarograms corresponding to the sequential addition of Zn or Cd (mol/mol Cd or Zn), to a solution containing the other cation, Cd or Zn  $5.5 \times 10^{-6}$  M, and an excess of  $\alpha$ -MT, in HEPES-phosphate buffer  $10^{-2}$  M at pH 8.0. (A) Addition of Zn to a solution containing Cd and 0.35 mol  $\alpha$ /mol Cd; (e) 0 mol Zn/mol Cd; (h) 0.99; (B) addition of Cd to a solution containing Zn and 0.4 mol  $\alpha$ /mol Zn; (c) 0 mol Cd/mol Zn; (d) 0.45; (e) 0.99; (f) 0.27, (g) 0.45.

influence of one cation on the complexing properties of the other and of comparing this behaviour with results obtained with binary and tertiary mixtures. Several examples of the evolution of polarograms are given in Fig. 6A and B, corresponding to the addition of zinc to a mixture containing Cd: $\alpha$  at 1:0.35 (Fig. 6A), and the addition of cadmium to a mixture Zn: $\alpha$  1:0.4 (Fig. 6B). In both cases a slight excess of  $\alpha$  is present in the initial dissolution.

The addition of zinc to the last obtained polarogram of  $\alpha$  addition to a Cd<sup>2+</sup> solution, exhibiting  $\alpha$ (Cd) and Cd $\alpha$  peaks (Fig. 6A(e)), provokes the gradual transformation of the Cd $\alpha$  peak into the Cd $\alpha'$  peak (Fig. 6A(h)). Peaks of Zn(II) reduction are detected, probably in the Zn $\alpha'$  peak and the Zn<sup>2+</sup> or Zn peak, the latter at a potential slightly more negative than that of free Zn<sup>2+</sup>. This Zn peak has already been detected during the study of some MTs [12,13,15,16] and of several mixtures FT:Cd:Zn [21,25]. The  $\alpha$ (Cd) peak is not affected by the zinc additions.

The effect of cadmium additions to a mixture containing Zn +  $\alpha$  is illustrated in Fig. 6B. The initial polarogram exhibits  $\alpha$ (Zn) and Zn $\alpha'$  peaks (Fig. 6B(c)). The addition of cadmium produces the diminishing and then the disappearance of  $\alpha$ (Zn) and the increase of  $\alpha$ (Cd) and of the reduction of Cd(II) in the form Cd $\alpha'$ . In addition the response due to the Zn(II) reduction increases, even the total zinc concentration remains unchanged, and the splitting of the initial peaks into Zn $\alpha'$  and, probably, Zn occurs.

The variation of peak currents of all species as a function of metal concentration added is represented in Fig. 7 with the aim of comparing obtained results. It is observed that the addition of Zn (Fig. 7a and c) does not affect the  $\alpha$ (Cd) intensity which remains constant; however, Cd $\alpha$  linearly decreases and then disappears at a proportion of  $\alpha$ :Cd:Zn, 1:3:1 (Fig. 7a) and 1:3:0.5 (Fig. 7c) and responses due to Cd $\alpha'$  and Zn $\alpha'$  increase with similar intensity until reaching the same value of  $\alpha$ (Cd) current. The detection of Zn<sup>2+</sup> or Zn occurs at a  $[Zn]_{ad}$  less than that required for obtaining the stability of polarographic responses. The final feature, when metal ions are in excess is the same:  $\alpha$ (Cd), Cd $\alpha'$  and Zn $\alpha'$  as that obtained when both cations are added together and are in excess (Fig. 4).

In the case of cadmium additions the most significant transformation occurs at different concentrations of cadmium in the two experiments (Fig. 7b and d). The final feature for binary mixtures  $\alpha$  + Zn is the same with peaks  $\alpha$ (Zn), Zn $\alpha'$  and Zn $\alpha$ , the latter, with a small intensity is only found in the case of addition of Zn<sup>2+</sup> to the  $\alpha$ -domain (Fig. 7b).  $\alpha$ (Cd) peak increases until a concentration of  $[Cd]_{ad}$  either  $> 6$  (Fig. 7b) or  $> 1$  (Fig. 6d).  $\alpha$ (Zn) decreases and disappears at  $[Cd]_{ad} > 4$  in the case of mixtures  $\alpha$  + Zn (Fig. 7b), but for Zn +  $\alpha$  the needed concentration

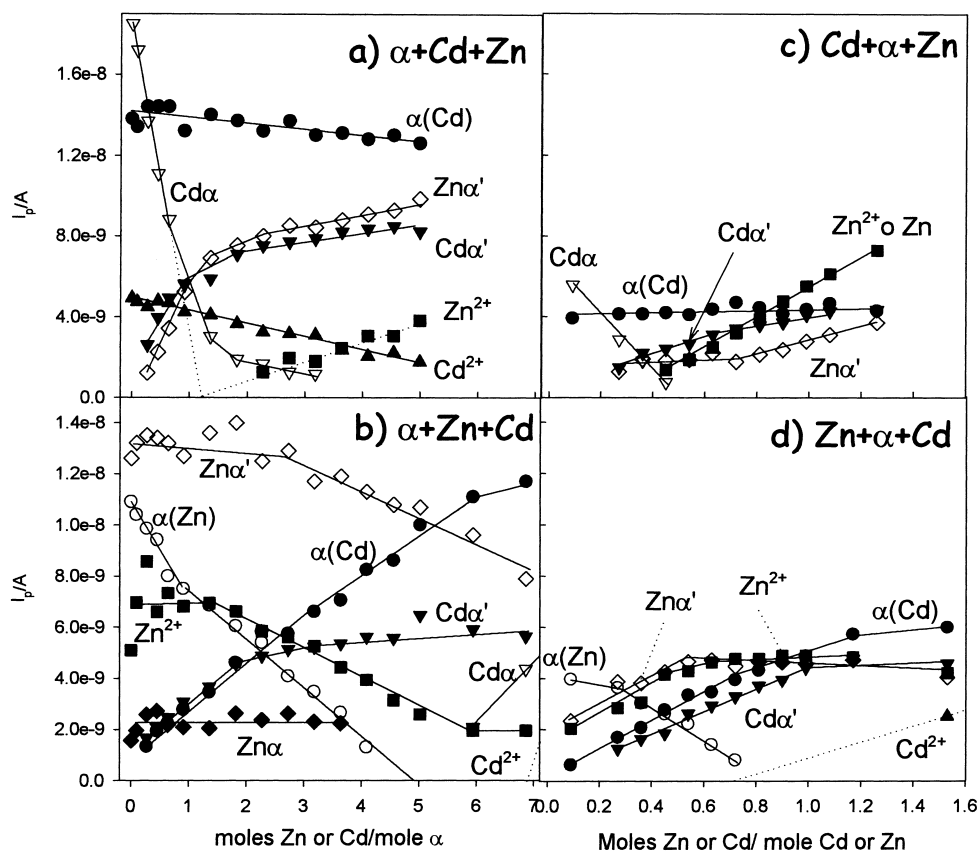


Fig. 7. Peak currents for all species obtained when Zn or Cd were added gradually to a solution containing both  $\alpha$ -MT  $5.5 \times 10^{-6}$  M and an excess of the other cation, Cd or Zn (a and b, respectively) or one cation, Cd or Zn, and an excess of  $\alpha$  (c and d, respectively), in HEPES-phosphate buffer  $10^{-2}$  M at pH 8.0.

of  $[\text{Cd}]_{\text{ad}}$  is smaller, leading to a different proportion of cations at the end of additions  $\alpha:\text{Cd}:\text{Zn} \approx 1:4:3$  and  $1:2.5:1$ , respectively.  $\text{Cd}\alpha'$  peak, the only peak detected for the Cd(II) reduction, increases with a similar slope to that of  $\alpha(\text{Cd})$  and reaches a plateau at  $[\text{Cd}]_{\text{ad}} > 2$  (Fig. 7b) or  $[\text{Cd}]_{\text{ad}} > 1$  (Fig. 7d). At great excess of  $[\text{Cd}]_{\text{ad}} > 5$ ,  $\text{Cd}\alpha$  peak starts to be detected and its intensity reaches a constant value at  $[\text{Cd}]_{\text{ad}} > 8.5$ . The fact that the  $\text{Zn}\alpha'$  intensity continuously decreases during the Cd additions (Fig. 7b) suggests that the Cd(II) can replace the Zn(II) initially bound to the  $\alpha$ -domain, in agreement with the values of complexing constants and, hence, the  $\text{Cd}\alpha$  peak attributed to a pure complex is detected at a great excess of cadmium.

### 3.4. Comparison between the polarographic results and those obtained using electrospray mass spectrometry

Several determinations of the stoichiometry of compounds in different binary mixtures  $\alpha:\text{Cd}$  and  $\alpha:\text{Zn}$  and tertiary mixtures  $\alpha:\text{Cd}:\text{Zn}$  at different metal ions to the  $\alpha$ -domain proportions have been performed by our team using electrospray ionisation mass spectrometry (ESI-MS) [33] with the aim of comparing results obtained using two different methods. The experimental conditions using both methods are not identical because the polarographic measurements were performed in each solution after sequential additions of one compound to another, with a slight variation of the

chemical composition, whereas in the case of MS measurements, several mixtures of a given composition have been prepared and immediately measured. Likewise, it should be pointed out that the MS signals for Cd $\alpha$  complex(es) are better defined than for Zn $\alpha$  complex(es), particularly in the case of tertiary mixtures for which both responses are simultaneously obtained; therefore the composition of zinc compounds and even their presence cannot be determined in a precise way.

The MS results are summarised as follows in order to associate polarographic peaks to different complexes:

$\alpha$ :Cd	1:0 to 1:5	Cd $\leq$ 3, Cd $_4\alpha$ Cd $\geq$ 4, Cd $_4\alpha$ > Cd $_5\alpha$
$\alpha$ :Zn	1:0 to 1:5	Zn $\leq$ 4, Zn $_3\alpha$ < Zn $_4\alpha$ Zn = 5, Zn $_4\alpha$
$\alpha$ + [Cd + Zn]	1:1:1	Cd $_3$ Zn $_1\alpha$ , Cd $_2$ Zn $_2\alpha$ , Cd $_1$ Zn $_2\alpha$
	1:2:2	Cd $_4\alpha$ , Cd $_3$ Zn $_1\alpha$ , Cd $_2$ Zn $_2\alpha$ , Cd $_1$ Zn $_2\alpha$ , Zn $_3\alpha$
	1:3:3	Cd $_4\alpha$ , Cd $_3$ Zn $_1\alpha$ , Cd $_2$ Zn $_2\alpha$
	1:4:4	Cd $_4\alpha$ , Cd $_3$ Zn $_1\alpha$
	1:5:5	Cd $_4\alpha$ , Cd $_3$ Zn $_1\alpha$

In the case of mixtures  $\alpha$ +Cd+Zn and  $\alpha$ +Zn+Cd all kinds of complexes can be found, particularly Cd $_4\alpha$  and mixed cadmium and zinc complexes. Depending on the order of addition as well as on relative proportions of three compounds, several complex(es) are prevalent in relation to others.

From MS results the assignment of polarographic peaks to possible complexes could be carried out, particularly in the case of binary mixtures because polarograms and mass spectra are simple and well defined.

For the binary mixtures  $\alpha$ +Cd and Cd+ $\alpha$  (Fig. 2a and c, Fig. 4) the only peak of the Cd(II) reduction detected, Cd $\alpha$ , should correspond to a Cd $_4\alpha$  complex in an excess of  $\alpha$ -domain and to a mixture of Cd $_4\alpha$  and Cd $_5\alpha$  when the cation is in excess, with peak potentials very similar. For the  $\alpha$ +Zn and Zn+ $\alpha$  mixtures (Fig. 2b and d, Fig. 4), two polarographic responses are detected, Zn $\alpha$  < Zn $\alpha'$  (Fig. 2b) in excess of ligand and only one, Zn $\alpha'$ , in excess of cation (Fig. 2d). Zn $\alpha'$  peak could be associated to Zn $_4\alpha$  complex. Like polarographic potentials of Zn(II) reductions are very close to each other, a discrimination of different complexes is difficult.

In the case of addition of both cations together to the  $\alpha$ -domain or the inverted additions, two different conditions can be distinguished (Fig. 4): an excess of ligand with the polarographic peaks:  $\alpha$ (Cd),  $\alpha$ (Zn), Cd $\alpha$  and Zn $\alpha'$  and an excess of metal ions with polarographic features:  $\alpha$ (Cd), Cd $\alpha'$  and Zn $\alpha'$ . These conditions can be compared to mixtures  $\alpha$ :Cd:Zn 1:1:1 (Fig. 4c and d, M/ $\alpha$  < 2 and Fig. 4g and h,  $\alpha$ /M > 0.5) with an excess of ligand, the MS results correspond to mixed complexes Cd $_3$ Zn $_1\alpha$ , Cd $_2$ Zn $_2\alpha$  and Cd $_1$ Zn $_3\alpha$ . For mixtures  $\alpha$ :Cd:Zn 1:2:2, corresponding to M/ $\alpha$  = 4 and  $\alpha$ /M = 0.25 in the Fig. 4, the

complexes identified using MS are Cd $_4\alpha$ , Cd $_3$ Zn $_1\alpha$ , Cd $_2$ Zn $_2\alpha$ , Cd $_1$ Zn $_3\alpha$  and Zn $_3\alpha$ , that means pure and mixed complexes with polarographic peaks Zn $\alpha'$  and Cd $\alpha'$ . When the metal ions are in excess (Fig. 4c and d, M/ $\alpha$  > 4 and  $\alpha$ /M < 0.25) with the same polarographic peaks the MS results lead to Cd $_4\alpha$ , Cd $_3$ Zn $_1\alpha$  and Cd $_2$ Zn $_2\alpha$ . Therefore, from these results the assignment of a Cd $\alpha$  polarographic peak to a pure compound, e.g. Cd $_4\alpha$  is not fully justified; however the attribution of Cd $\alpha'$  to mixed cadmium and zinc complexed with the  $\alpha$ -domain is very probable. In addition, similar values of Zn $\alpha'$  and Cd $\alpha'$  peak intensities, when metal ions are in excess (Fig. 4c) confirm the attribution of these peaks to the same compound containing both cations. Comparing the attribution of polarographic Cd(II) and Zn(II) reduction peaks of complexes with the  $\beta$ -domain [34] the study of  $\alpha$ -domain complexes is more ambiguous, probably due to the different kinetics of complexation for both  $\alpha$  and  $\beta$ -domain [3].

At this stage it is worth clarifying that, in the case of cadmium complexes, both the electrochemical and MS responses are well defined allowing one to clearly distinguish three polarographic signals, Cd $^{2+}$ , Cd $\alpha'$

and  $Cd\alpha$  as well as between pure and mixed complexes using MS results. Concerning Zn compounds the polarographic peaks are very close to each other and the corresponding signals in the MS spectra are also poorly defined. Nevertheless, it seems to be clear that several stable forms of cadmium and zinc complexation with the  $\alpha$ -domain co-exist, depending on the ratio between the  $\alpha$ -domain and metal ions, the proportion of cadmium to zinc and the order and mode of additions of one compound to another.

### 3.5. Estimation of apparent stability constants $\alpha$ -M

The apparent stability constants of  $Cd\alpha$ ,  $Zn\alpha$  and  $Hg\alpha$  complexes have been evaluated using previously described approaches [8,18,19]. In the case of  $Cd\alpha$  and  $Zn\alpha$  constants the differences between the potential of free cations and the complexed ions have been taken from polarograms recorded in the case of binary mixtures. For the evaluation of  $Hg\alpha$  constants the difference of potentials between the oxidation of mercury in the supporting electrolyte (unlimited current in oxidation) and the oxidation of the mercury in the presence of the  $\alpha$ -domain alone was used. The concentration of the ligand, included in the stability constants value, was taken as being approximately  $10^{-5}$  M.

The  $\log K'_{Cd\alpha}$ ,  $\log K'_{Zn\alpha}$  and  $\log K'_{Hg\alpha}$  values are 14, 10 and 31, respectively. These values are comparable to those obtained for Cd, Zn MT of different origin [8,9,11] and for M-FT complexes [19–22] as well as for M- $\beta$ -domain complexes [34], M being Cd, Zn or Hg. Even if the assumption on which this estimation is based can be considered as only approximate, the stability constants of the  $\alpha$ -domain of MT with different metal ions have been estimated here for the first time.

## 4. Conclusions

DPP, under experimental conditions which ensure that the interaction phenomena between the electrode and the compounds are avoided, appears to be a good technique for monitoring changes of different species in solution and for providing information about complexation properties. It allows one to make a clear distinction between free cations,  $Cd^{2+}$  and  $Zn^{2+}$ , and these metal ions complexed with the  $\alpha$ -domain of MT, and consequently allows one to follow the evolution

of cadmium and zinc complexes simultaneously. Few analytical techniques can be used for obtaining information, at the same time, of both complexes with similar responses, such as is the case of the reduction of cadmium and zinc at the mercury electrode based on analogous electrochemical mechanisms.

The  $\alpha$ -domain of MT is capable of complexing metal ions, cadmium and zinc, at a proportion of three five-metal ions and one organic molecule in the case of binary mixtures  $\alpha + Cd$  and  $\alpha + Zn$ . Pure cadmium and zinc complexes,  $Cd_4\alpha$  and  $Cd_5\alpha$  and  $Zn_3\alpha$  and  $Zn_4\alpha$ , depending on proportions of metal ions to the  $\alpha$ -domain, have been confirmed using MS techniques. The same ratio of the  $\alpha$ -domain to metal ions has been obtained when both cations are simultaneously present leading to tertiary mixtures  $\alpha + Cd + Zn$ ; however the formation of mixed cadmium and zinc complexes prevails for tertiary mixtures which has been verified by the use of MS results. In fact the polarographic features are not the same for binary mixtures as for tertiary mixtures. Two different electrochemical responses of the reduction of Cd(II) and Zn(II) complexed with the  $\alpha$ -domain have been found and attributed to two different forms of complexation of cadmium and zinc with the ligand. The obtention of one of the two responses or both together depends on the relative proportion of cations. Whereas in the case of cadmium complexes the distinction of  $Cd^{2+}$ ,  $Cd\alpha'$  and  $Cd\alpha$  polarographic peaks is unambiguous, for the zinc compounds the resolution of different signals is reached using a mathematical approach.

When one metal ion, cadmium or zinc, is added to a solution of pure complexes  $Zn\alpha$  and  $Cd\alpha$ , a reorganisation in the molecules is produced when the metal ion is incorporated by successive additions leading to the transformation of pure complexes into mixed complexes. This is displayed by a gradual transformation of the  $Cd\alpha$  peak into the  $Cd\alpha'$  when zinc is added to a  $Cd + \alpha$  mixture or the appearance and gradual increase of the  $Cd\alpha'$  peak when cadmium is added to a  $Zn + \alpha$  mixture, obtained in the electrochemical study.

Taking into account the values of the apparent stability constants of  $Cd\alpha$  and  $Zn\alpha$  complexes, added cadmium should remove the zinc initially bound to the  $\alpha$ -domain as was reported in several papers. However, the pH of experiments used in most of these works are close to neutral pH and taking into account that the formation of the  $Zn\alpha$  complex starts at  $pH > 6$ , the

stability constant at a pH close to 6 should be low and hence zinc could easily be removed by the added cadmium. In our work the experiments were performed at pH 8, at which the value of the complexing constant is  $\log K_{Zn\alpha} \approx 10$ , the incorporation of cadmium is produced without the displacement of zinc, confirmed by both polarographic and MS results.

The electrochemical method does not allow one to get information about structural features and hence to know if the incorporation of metal ions to a formed complex(es) occurs either with the same kind of co-ordination or if another kind of co-ordination is induced, but allows one to note important changes in the polarographic features for given Cd/Zn ratios as well as to observe the combination of given responses under different experimental conditions.

The use of another completely different technique from electroanalytical methods such as MS from which information of the molecular weight of compounds and the stoichiometry of complexes can be derived has been very useful for the assignment of different cadmium and zinc electrochemical responses as well as for supporting our assumptions about the formation of, at least, two different forms of complexation of cadmium and zinc with the  $\alpha$ -domain of MT.

To our knowledge this paper constitutes the first study on the complexing properties of the  $\alpha$ -MT domain using electrochemical methods and complements another similar work using the  $\beta$ -MT domain.

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## References

- [1] J.H.R. Kägi, M. Nordberg (Eds.), *Metallothionein*, Birkhauser, Basel, 1979.
- [2] J.H.R. Kägi, Y. Kogima (Eds.), *Metallothionein II*, Birkhauser, Basel, 1987.
- [3] M.J. Stillman, C.F. Shawn III, K.T. Suzuki (Eds.), *Metallothioneins, Synthesis, Structure and Properties of Metallothioneins, Phytochelatins and Metal-Thiolate Complexes*, VCH, New York, 1992.
- [4] J.F. Riordan, B.L. Vallee (Eds.), *Methods of Enzymology*, Vol. 205, *Metallobiochemistry, Part B, Metallothioneins and Related Molecules*, Academic Press, London, 1991.
- [5] K.T. Suzuki, N. Imura, M. Kimura (Eds.), *Metallothionein III, Biological Roles and Medical Implications*, Birkhauser Verlag, Basel, 1993.
- [6] G. Bordin, A.R. Rodríguez (Eds.), *Metallothioneins (special issue)*, *Talanta*, Vol. 46, 1998.
- [7] C.D. Klaassen (Ed.), *Metallothionein IV*, Birkhauser Verlag, Basel, 1999.
- [8] A. Muñoz, A.R. Rodríguez, *Electroanalysis* 7 (1995) 674.
- [9] C. Ruiz, J. Mendieta, A.R. Rodríguez, *Anal. Chim. Acta* 305 (1995) 285.
- [10] C. Ruiz, A.R. Rodríguez, *Anal. Chim. Acta* 325 (1996) 43.
- [11] C. Ruiz, A.R. Rodríguez, *Anal. Chim. Acta* 350 (1997) 305.
- [12] O. Nieto, G. Bordin, A.R. Rodríguez, G. Hellemans, M. DeLey, *Talanta* 46 (1998) 315.
- [13] O. Nieto, G. Bordin, A.R. Rodríguez, G. Hellemans, M. DeLey, *Bioelectrochem. Bioenergetics* 46 (1998) 45.
- [14] C. Harlyk, O. Nieto, G. Bordin, A.R. Rodríguez, *J. Electroanal. Chem.* 458 (1998) 199.
- [15] M. Dabrio, A.R. Rodríguez, *Anal. Chim. Acta* 385 (1999) 295.
- [16] M. Dabrio, A.R. Rodríguez, *Anal. Chim. Acta*, 406 (2000) 171.
- [17] M. Dabrio, Rodríguez A.R., *Electroanalysis*, in press.
- [18] J. Mendieta, J. Chivot, A. Muñoz, A.R. Rodríguez, *Electroanalysis* 7 (1995) 663.
- [19] A. Muñoz, A.R. Rodríguez, *Electroanalysis* 7 (1995) 670.
- [20] J. Mendieta, A.R. Rodríguez, *Electroanalysis* 8 (1996) 473.
- [21] O. Nieto, A.R. Rodríguez, *Bioelectrochem. Bioenergetics* 40 (1996) 215.
- [22] C. Harlyk, G. Bordin, O. Nieto, A.R. Rodríguez, *Electroanalysis* 9 (1997) 608.
- [23] C. Harlyk, G. Bordin, O. Nieto, A.R. Rodríguez, *J. Electroanal. Chem.* 446 (1998) 139.
- [24] C. Harlyk, G. Bordin, O. Nieto, A.R. Rodríguez, *J. Electroanal. Chem.* 451 (1998) 267.
- [25] O. Nieto, A.R. Rodríguez, *Electroanalysis* 11 (1999) 175.
- [26] M. Esteban, C. Harlyk, A.R. Rodríguez, *J. Electroanal. Chem.* 468 (1999) 202, and references therein.
- [27] A.J. Zelazowski, J.A. Szymanska, A.Y.C. Law, M.J. Stillman, *J. Biol. Chem.* 259 (1984) 12960.
- [28] M.J. Stillman, W. Cai, A.J. Zelazowski, *J. Biol. Chem.* 262 (1987) 4538.
- [29] S. Matsumoto, S. Nakayama, Y. Nishiyama, Y. Okada, K. Min, S. Onasaka, K. Tanaka, *Chem. Pharm. Bull.* 40 (1992) 2694.
- [30] Y. Li, U. Weser, *Inorg. Chem.* 31 (1992) 5526.
- [31] N. Cols, N. Romero-Isart, M. Capdevila, B. Oliva, P. González-Duarte, R. González-Duarte, S. Atrian, *J. Inorg. Biochem.* 68 (1997) 157.
- [32] S. Klauser, Universität Zürich, personal communication
- [33] M. Dabrio, G. Van Vyncht, G. Bordin, A.R. Rodríguez, *Anal. Chem.*, in press.
- [34] M. Dabrio, A.R. Rodríguez, *Analisis*, 28 (2000) 20.