Characterization of horse kidney metallothionein isoforms by electrospray MS and reversed-phase HPLC-electrospray MS

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Received 22nd June 1998, Accepted 25th August 1998

Pneumatically assisted electrospray mass spectrometry (ESMS) in the direct mode and as a chromatographic detection technique was developed for the characterization of horse kidney metallothionein isoforms. Direct analysis in an acidic medium showed the presence of three major and five minor isoforms, the molecular masses of which were determined. The presence of the major isoforms (two of which matched the molecular masses calculated according to the published sequences) was confirmed by complexation with Cd at pH 4.0 and the determination of the stoichiometry of the complexes formed. Reversed-phase chromatography of Cd–MT complexes (pH 6.0) gave two signals corresponding to the MT-1A and MT-1B isoforms. A post-column acidification procedure was developed to eliminate the possibility of artefacts associated with the formation of mixed-metal (Cd, Zn) complexes during chromatography in neutral media, and to improve the accuracy of the determination of the molecular mass of MT isoforms.

Introduction

Metallothionein (MT), a polymorphic non-enzymatic low molecular mass (6–7 kDa) metal-binding protein, was first isolated from horse kidney in 1957,1 and since then it has been the subject of numerous studies owing to its putative role in homeostatic control, metabolism and detoxification of a number of trace metals, especially cadmium and zinc.2–4 An important aspect of these studies has been the mechanisms involved in MT gene expression5 that lead to the MT polymorphism consisting of the variation of the primary structure of MT by the substitution of 1–15 amino acids. This leads to the formation of MT isoforms and, if the amino acid heterogeneities are minor, subisoforms.6 Studies of the polymorphism of horse kidney metallothionein fall into two basic categories. The first includes methods based on the isolation and purification of the individual isoforms followed by their sequencing, e.g., by Edman degradation.7 This method is tedious and time consuming but allows the identification of four horse kidney metallothionein isoforms, the structures of which are summarized in Fig. 1.8

Another approach is based on analytical reversed-phase chromatography9–13 and capillary zone electrophoresis (CZE),14,15 the resolution of which is sufficient to detect amino acid microheterogeneities leading to small differences between the individual isoforms in terms of the retention or migration time, respectively.16 This approach has led to speculative, confusing and often contradictory data because of the virtual impossibility of knowing what species was detected. Indeed, the chromatograms (electropherograms) reported showed different morphologies, despite the use of apparently identical analytical techniques and operating conditions (column, mobile phase).9,10,13 and differ in terms of the number of peaks observed.

Electrospray mass spectrometry (ESMS), owing to its speed and precision (±1 Da) of molecular mass determination, has been shown to be an attractive technique for the characterization of polypeptides17,18 and for studies of non-covalent metal ion–protein interactions.19 ESMS with pH control has been used for the determination of metals in native and reconstituted metallothioneins, showing how many and what cations are incorporated per molecule of metallothionein.20–22 A deeper insight into the polymorphism of metallothionein can be gained by HPLC with ESMS detection of the eluting species.22–24 LeBlanc optimized the HPLC of a Cd3Zn3MT preparation and showed the possibility of the detection of Cd and Zn by ionspray MS operated in the source collision induced dissociation mode.23 All the above studies were limited to rabbit liver metallothionein and, in particular, to one of its isoforms, MT-2.20,24

The objective of this study was to evaluate pneumatically assisted ESMS and reversed-phase (RP) HPLC with ESMS detection to characterize the potentially present isoforms of the horse kidney metallothionein. RP-HPLC in neutral media suffers from artefacts related to the formation of mixed metal (especially Cd–Zn) complexes; a post-column acidification procedure was developed to eliminate this type of artefact.

Experimental

Instrumentation

HPLC was performed using an ABI 140C microbore syringe pump, an ABI Model 112A injection module and an ABI Model 785A absorbance detector equipped with a microbore cell (Applied Biosystems, Foster City, CA, USA). ESMS experiments were performed using a Perkin-Elmer SCIEX (Thornhill, ON, Canada) API 300 ionspray triple-quadrupole mass spectrometer. Analyte solutions were introduced into the source by means of a 500 μl Hamilton (Reno, NV, USA) gas tight syringe and a Harvard Apparatus (South Natick, MA, USA) Model 55111 syringe pump. BioToolBox software was used for the calculation of molecular masses and deconvolution of protein mass spectra.
Reagents and standards

Methanol and acetonitrile (Sigma-Aldrich, St. Louis, MO, USA) were of LC grade. Water purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used throughout. Liquid nitrogen (99.996%) was evaporated in situ and supplied at 40 lb in$^2$. The buffer solution was prepared by dissolving 5 mmol L$^{-1}$ of ammonium acetate in water (or in 50% acetonitrile) and adjusting the pH to 6.0 with acetic acid. The buffers were sparged with helium to remove dissolved oxygen and thus to attain a non-oxidizing environment. The latter is necessary in view of the observed susceptibility of MTs to oxidation during isolation.12

Metallothionein preparation isolated from horse kidney containing, according to the specification, a mixture of two isoforms, MT-1A and MT-1B (Lot 37H9544), was purchased from Sigma (Saint Quentin Fallavier, France). It was found (by ICP-MS) to contain 4.8% Cd, 0.8% Zn and 0.9% Cu. Batch-to-batch variations can be significant; the preparations should be checked for purity and fully characterized prior to their use as standards.11

A stock standard MT solution (1 mg mL$^{-1}$) was prepared by dissolving 1 mg of metallothionein in 1 mL of water. Working standard solutions were prepared by the dilution of the stock standard solution with water or buffer as required. The stock standard solution was kept in a refrigerator at 4 °C in the dark.

HPLC conditions

Separations were carried out using a Vydac C$_8$ 150 mm × 1 mm id × 5 µm film thickness column. The pump flow rate was set at 40 µL min$^{-1}$, which corresponded to a pressure of 4.5 MPa. The injection volume was 5 µL. MT isoforms were eluted with a linear gradient of acetonitrile (10–16% B) within 50 min; buffer A was 5 mM acetate buffer in water (pH 6.0) and buffer B was 5 mM acetate buffer in CH$_3$CN–H$_2$O (1 + 1) (pH 6.0). The solutions were de-gassed by sparging with helium.

For post-column acidification, a flow of a mixture of formic acid–methanol (30 + 70 v/v) at 4 µL min$^{-1}$ was mixed via a zero-dead volume T-piece (Interchim, Montluçon, France) in order to obtain a final pH of 1.9 and to obtain apo-MT isoforms on arrival at the detector. A mixing coil (25 cm × 250 µm id, made of PEEK) was placed behind the T-piece to accomplish the mixing process. Without this tube, the demetallation of the Cd$_2$–MT complexes turned out to be irreproducible and incomplete; Cd$_4$-MT were occasionally present.

Electrospray MS conditions

Direct analysis. An MT solution was introduced at 5 µL min$^{-1}$ via a fused silica capillary (100 µm id). Mass spectra were acquired with an ES voltage of 4400 V and an orifice potential of 60 V. A total of 10 scans were measured with a step size of 0.5 Da and a dwell time of 5 ms. The m/z ranges scanned by Q1 were 800–1800 for apo-MT isoforms, 700–1800 for Cd$_2$–MT complexes and 1200–1800 for Cd$_4$–MT complexes. Q2 and Q3 were operated in the rf-mode only. The duration of the run was 1.6, 1.8 and 1 min for apo-MT, Cd$_4$–MT and Cd$_2$–MT, respectively. Apo-MT isoforms were obtained in a solution (pH 1.8) of 25 mM HCl in 30% v/v aqueous methanol. Solutions at pH 4.0 (Cd$_4$–MT) and 6.0 (Cd$_2$–MT) were prepared in 5 mM ammonium acetate buffer in 30% v/v methanol adjusted to the require pH with concentrated acetic acid.

HPLC-ESMS analysis. An m/z range of 1200–1800 was monitored, which allowed the observation of the +4 and +5 ionization states of the metallated isoforms and of the apo-MTs (in the case of the post-column dilution). The mass spectra were acquired with a step size of 0.5 Da and a dwell time 2 ms, which required 2.8 s per scan.

Results and discussion

Sequences of four horse kidney MT isoforms, MT-1A, MT-1L54 (heterogeneity L/S of MT-1A), MT-1R39 (heterogeneity R/G of MT-1A) and MT-1B have been reported in the literature (Fig. 1).8 All of them contain 62 amino acids. Roman numbers indicate the sites of fixation of cadmium by the thiol groups of the cysteine residues in each of the clusters α and β. The acetyl terminal group at the end of the chain is characteristic of vertebrates. The sequences reported allowed the calculation of the molecular masses of the apo-isoforms and tentative molecular masses (M) of the Cd$_{1}$– and Cd$_{2}$–MT complexes according to the equation $M_{(Cd,x-MT)} = M_{(apo-MT)} + xM_{Cd}$

Fig. 1 Amino acid sequences of horse kidney metallothioneins described in the literature.$^8$ (a) Amino acids that are part of the β cluster with three Cd atoms; (b) amino acids that are part of the α cluster with four Cd atoms.
2 $\times M_{Rt}$, where $x = 4$ or 7. The molecular mass values are summarized in Table 1.

Characterization of horse kidney metallothionein isoforms by ESMS in the direct mode

Analyses under demetallating conditions. At pH 1.8, Cd and Zn are dissociated from the metallothionein, giving rise to the presence of an apo-metallothionein in the analyte solution. Fig. 2 shows an electrospray mass spectrum obtained under these conditions. The different MT isoforms are observed in four ionization states (+7, +6, +5 and +4), the most intense being +4. The similar patterns observed for the different charge states indicate the presence of several isoforms. Except for the metallothionein envelope, the spectrum indicates the presence of at least three other proteins: (i) $M = 12,005.4 \pm 0.8$ Da (ionization states +14, +13, ... +9, +8), tentatively identified as cytochrome $c$ having a molecular mass close to 12 kDa (horse kidney cytochrome $c$ has a molar mass of 12,384); (ii) $M = 8295 \pm 0$ Da (ionization states +6 and +5); this protein was observed in the purified preparations of rabbit liver metallothionein but remains unidentified; and (iii) $M = 8180.5 \pm 0.5$ Da (ionization states +6 and +5); the presence of this protein was also noted in the unpurified preparation of rabbit liver metallothionein.

The data in Fig. 2 allowed the reconstruction of the $m/z$ spectrum to obtain a true mass spectrum of the horse kidney metallothionein, shown in Fig. 3. The presence of eight putative isoforms can be observed, three of which can be considered major ($\alpha$, $\beta$, $\delta$) while the remaining five ($\gamma$, $\epsilon$, $\zeta$, $\eta$, $\theta$) are minor. The molecular masses of these putative isoforms are given in the figure.

The signals denoted by $\alpha$ and $\zeta$ can be assigned to the isoforms apo-MT-1A ($M = 6082.3$) and apo-MT-1R39 ($M = 6181.4$) (heterogeneity R/G of MT-1A), respectively. Regarding the peak $\beta$ there remains an ambiguity about attributing it to the form MT-1B ($M = 6109.3$) or MT-1L54 ($M = 6108.3$) (heterogeneity L/S of MT-1A), which differ by only 1 mass unit. The precision of the determination of the molecular mass in the direct mode may be worse than the theoretical value of 0.7 Da since the analytical peak is not baseline resolved, apparently because of the presence of impurities of similar molecular masses.

Regarding the remaining five peaks, some hypotheses may be made regarding the exchange of one amino-acid with respect to the known isoforms. The form MT-$\gamma$ differs from the form MT-1B by 11.64 Da, which may correspond to the exchange I/T or L/T. The form MT-$\delta$ differs from MT-1L54 by 35.66 Da, which fits perfectly the H/T heterogeneity. The MT-$\epsilon$ signal differs from MT-1R39 by $-14.89$ Da, which may correspond to the I/Q or L/Q heterogeneities. No hypothesis could be made for the peaks denoted $\eta$ and $\theta$ in Fig. 3.

It can be therefore concluded that the preparation analysed is composed of the forms denoted $\alpha$ and $\beta$, which correspond well with the two isoforms MT-1A and MT-1B known in the literature. It cannot, however, be considered as a simple mixture of these forms as specified by Sigma since six other putative isoforms are present in addition to three other non-metallthionein-like proteins.

Table 1 Molecular masses of putative horse kidney metallothionein isoforms found by direct infusion ESMS and by HPLC with ESMS detection

<table>
<thead>
<tr>
<th>Compound</th>
<th>ESMS</th>
<th>HPLC–ESMS</th>
<th>Literature sequences</th>
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<tbody>
<tr>
<td></td>
<td>$M_{Rt}$</td>
<td>$M_{Rt}$</td>
<td>$M_{Rt}$</td>
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<tr>
<td>Compound</td>
<td>apo-MT</td>
<td>Cd4–apo-MT</td>
<td>apo-MT</td>
</tr>
<tr>
<td>MT-$\alpha$</td>
<td>6081 ± 1</td>
<td>6523 ± 1</td>
<td>442</td>
</tr>
<tr>
<td>MT-$\beta$</td>
<td>6107.2 ± 0.7</td>
<td>6548 ± 1</td>
<td>440.8</td>
</tr>
<tr>
<td>MT-$\delta$</td>
<td>6143 ± 1</td>
<td>6583 ± 1</td>
<td>440</td>
</tr>
</tbody>
</table>

Fig. 2 ES mass spectrum of a horse kidney metallothionein sample dissolved at 100µg mL$^{-1}$ in 25 mm hydrochloric acid in 30% aqueous methanol (pH 1.8) and infused into the ionspray source at 5 µl min$^{-1}$. Peaks are annotated with $m/z$ values and ionization states. The following envelopes can be seen: (a) protein with $M = 12,005.4 \pm 0.8$ Da; (b) horse kidney metallothionein; (c) protein with $M = 8180.5 \pm 0.5$ Da; and (d) protein with $M = 8295 \pm 0$ Da.

Analyst, 1998, 123, 2125–2130

2127
Analyses of metallothionein complexes with Cd and Zn. It has been reported that in moderately acidic (pH 4.0) and neutral (pH 6–7) media, the complexes of rabbit liver metallothionein with Cd and Zn\textsuperscript{21} retain their conformation in the gas phase and produce ions of intact complexes at the ES source. ES mass spectra of the horse kidney MT preparation at pH 4.0 and 6.0 are shown in Fig. 4(a) and 4(b), respectively. The spectra appear complex in comparison with those of a rabbit liver MT for two principal reasons: (i) the presence of other proteins detected above, the ionization of which does not seem to be affected by an increase in pH, and (ii) the evidently poorer response of the metallothionein complexes at pH 4.0 and, especially, at pH 6.0. This results in the MT signal being masked by signals from the foreign proteins.

Fig. 4 demonstrates that the impurity proteins are not MT related because they do not apparently show potential for complexing Cd or Zn. The molecular masses of the compounds are independent of pH, which indicates, unlike MT-like proteins, no change in their composition in terms of the metallation. At pH 6.0 [Fig. 4(b)], each of the proteins ($M = 8180.5$ and 8295.0) shows an intense signal within each charge state envelope followed by four or more peaks with diminishing intensity. These peaks probably correspond to adducts of water (18 Da separating two consecutive peaks of each envelope).

Despite the poorer ionization of the MT complexes, the data in Fig. 4 allow the reconstruction of the MT spectrum at pH 4 (not shown). The pattern of the reconstructed spectrum follows that of the spectrum of apo-MT. It allows the recognition of the MT-1B or MT-1L\textsuperscript{54} isoform and two smaller peaks (MT-\textalpha and MT-\textdelta) as Cd\textsubscript{4} complexes. The signals of other isoforms, if present, are lost in the noise. At pH 6.0, a large and poorly defined envelope of Cd\textsubscript{7}–MT species is observed in the +4 ionization state, the +5 ionization state is eclipsed by the impurity proteins. The molecular masses of the Cd\textsubscript{7}–MT species cannot be precisely determined.

**HPLC of horse kidney metallothionein with ESMS detection**

The ambiguity in the case of similar molecular masses of MT species due to the insufficient resolution of the mass spectra of apo-metallothioneins can be avoided by separating the species and calculating on-line the molecular masses of the eluting compounds. Since the horse kidney metallothionein preparation had been purified by size-exclusion and anion-exchange chromatography, separation by reversed-phase chromatography was attempted. Since the separations of apo-MT require the use of a 0.1% TFA (15 mM) buffer, which suppresses the ES signal, we chose to work with neutral buffers. Both the native peptide conformation and the original metal composition are preserved at neutral pH, while retention times are shorter and the recoveries are high (90%), in contrast to those observed with acidic buffers.\textsuperscript{12} A chromatogram obtained under optimized conditions is shown in Fig. 5. The chromatogram shows two
major peaks which are relatively broad, probably because they contain several unresolved species. There is a shoulder visible at the second major peak while some minor peaks precede the major peaks in the chromatogram. Similar patterns were obtained by Van Beek and Baars, and Richards and Beattie; in the latter case the peaks were not well resolved by HPLC at this pH but were by CZE. Klauser et al. demonstrated that horse kidney MT is comprised of at least three species differing in their amino acid compositions. The identification was based on the elution order (the first eluting species was termed MT-2 and the second MT-1) without obtaining proof of identity. Bordin et al. reported a reversed-phase chromatogram for a horse kidney metallothionein preparation similar to ours, with a major peak preceded by a small one, except for the presence of two or three minor peaks following the major signal. On the basis of the hydrophobicities of different MTs reported in the literature, the small peak was assigned to MT-1A and the major peak to MT-1L54. No peak from MT-1B was observed.

We attempted to investigate the identity of these signals using on-line ESMS detection. Two different analytical modes were evaluated, MS of metal complexes and MS of demetallated (by post-column acidification) metallothionein isoforms, as described below.

Detection of metal–MT complexes. Mass spectra taken at the apices of peaks 1 and 2 in the chromatogram (Fig. 5) are shown in Fig. 6(a) and (b), respectively. They show that the chromatographic peaks are not pure in terms of MS and each of them contains several chemically different species. On the basis of the molecular masses of the co-eluting species [cf. Fig. 6(a)], the peak 1 in Fig. 5 can be attributed to the isoform MT-α (or MT-1A) according to Bordin et al. The numerous peaks in the mass spectra result from the simultaneous presence of the mixed Cd–Zn–MT-1A complexes that can be precisely identified on the basis of their molecular masses. The mass spectrum [Fig. 6(b)] taken at the apex of peak 2 in the chromatogram shown in Fig. 5 also shows the presence of differently metallated species. The precision of the determination of the molecular mass, negatively affected by the fact that the molecular mass measured is not that of an apo-form but of a complex of ambiguous stoichiometry, does not allow certitude regarding the origin of the second peak in the chromatogram (it can be either MT-1B \((M = 6109.3)\) or MT-1L54 \((M = 6108.3)\) Da) (cf., Table 1).

The direct detection of the eluting horse kidney MT complexes by ESMS gives a simple and rapid opportunity for the identification of the species eluting from a chromatographic column. It suffers from several drawbacks, some of which can conceivably have different properties related to differences in structure. Richards and Beattie stated that the type of metal bound to MT does not appear to affect the isomorph migration time in CZE. By calculating the differences between the adjacent peaks, these interferences can be detected.

A more serious potential problem is due to the fact that the information on the molecular mass of the metallothionein is obtained indirectly after subtracting the molecular mass of seven Cd atoms \((-442.0)\) Da. However, Yu et al. and our experiments showed that the imprecision here may be \(\pm 2\) Da, which may be critical for the precise identification of a given isoform. Another problem is the loss of sensitivity during ES ionization of metalloforms, which, together with the complexity of the spectra may hamper the detection and analysis of minor chromatographic signals.

We attempted to eliminate these problems by a post-column acidification of the chromatographic eluent leading to the formation of apo-metallothionein following their separation as Cd complexes.

Detection of apo-metallothionein isoforms after post-column acidification. The fact that separation is carried out in acetonitrile at neutral pH makes the detection suffer from the lack of sensitivity, poor signal-to-noise ratio and the numerous artefacts due to the formation of mixed metal complexes that further eclipse the acquired mass spectra. Another disadvantage is that the molecular mass of the metallothionein isoform is back-calculated assuming a given stoichiometry of the metallated species which negatively affects the precision of the determination of the molecular mass of the apo-metallothionein isoform.

The shape of the TIC chromatogram obtained after post-column acidification (not shown) is similar to that in Fig. 5. The obtained. One of them is relevant to the analysis of preparation that contain both Cd and Zn, which can give rise to the presence of several MT species differing in their metal content (i.e., metalloforms). This means that the same isoform gives several signals in a mass spectrum. The metalloforms could conceivably have different properties related to differences in structure. Richards and Beattie stated that the type of metal bound to MT does not appear to affect the isomorph migration time in CZE. By calculating the differences between the adjacent peaks, these interferences can be detected.

Fig. 5 Chromatogram obtained for a solution of horse kidney metallothionein (500 µg mL\(^{-1}\)) by microbore reversed-phase HPLC with UV detection (\(\lambda = 254\) nm); 2.5 µg injected. Gradient: 5–8% CH\(_3\)CN within 50 min. For identification of peaks 1 and 2, see Fig. 6 and the text.

Fig. 6 Mass spectra taken at the apices of the peaks shown in Fig. 5. (a) Peak 1; (b) peak 2. Peaks are annotated with \(m/z\) values; formulae of the putative complexes were attributed and are shown. HK denotes horse kidney metallothionein.
introduction of the post-column dead volume in the mixing coil does not have any noticeable effect on the peak width. Fig. 7(a) and (b) show mass spectra taken at the apices of the chromatographic peaks 1 and 2, respectively. As expected, the mass spectra are simpler (containing fewer signals for each of the chromatographic peaks) and show a distinctly better signal-to-noise ratio.

The mass spectrum taken at the apex of peak 1 confirms unambiguously that this peak represents the isoform MT-1A ($M = 6082.2 \pm 0.2$ found against 6082.3 calculated from the sequence). The spectrum indicates an impurity of this peak ($M = 6113.2 \pm 0.7$). The origin of this peak is unknown. It could not be detected in Figs. 2 and 6(a) because of the large noise from the apo-MT-$\beta$ form in Fig. 2 and from the background in Fig. 6(a). It may be an ES artefact but no satisfactory premise for this hypothesis was found either.

The mass spectrum taken at the apex of peak 2 allows the calculation of the molecular mass of the MT isoform purified on-line by RP-HPLC. The value found, $6109.5 \pm 0.8$ Da, matches closely the value of 6109.3 calculated on the basis of the sequence of MT-1B. This is in contradiction to Bordin et al.,13 who, on the basis of the hydrophobicity of amino acids for this hypothesis was found either.

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![Fig. 7](image-url) Mass spectra taken at the apices of the peaks shown in Fig. 5 after post-column acidification as described under Experimental. (a) Peak 1; (b) peak 2. Peaks are annotated with $m/z$ values; formulae of the putative complexes were attributed and are shown. HK denotes horse kidney metallothionein.

**Conclusions**

Many problems related to the characterization of horse kidney metallothionein have been arisen owing to the virtual impossibility of identifying the signals obtained in RP-HPLC and CZE, the only techniques able to distinguish between the isoforms with amino acid microheterogeneities. These problems can be solved by using ESMS, which appears to be an attractive technique for the identification of metallothionein isoforms. The selectivity can be improved by using this technique for on-line detection of the species eluting from a chromatographic column. Whereas the separation is realized in neutral media, post-column acidification is required to eliminate the artefacts due to the existence of several mixed metallocomplexes of the same isoform, which complicates the identification of the particular isoforms.

**References**


*Paper 8/04713H*