Potential for Using Isotopically Altered Metalloproteins in Species-Specific Isotope Dilution Analysis of Proteins by HPLC Coupled to Inductively Coupled Plasma Mass Spectrometry

ARTICLE *in* ANALYTICAL CHEMISTRY · AUGUST 2005

Impact Factor: 5.64 · DOI: 10.1021/ac050256t · Source: PubMed

4 AUTHORS, INCLUDING:

Chris Harrington
Royal Surrey County NHS Foundation Trust
95 PUBLICATIONS **1,474** CITATIONS
SEE PROFILE

Michael J Watts
British Geological Survey
61 PUBLICATIONS **448** CITATIONS
SEE PROFILE

Anal. Chem. **2005,** *77,* 4034−4041

Potential for Using Isotopically Altered Metalloproteins in Species-Specific Isotope Dilution Analysis of Proteins by HPLC Coupled to Inductively Coupled Plasma Mass Spectrometry

Chris F. Harrington,*,† Daniel S. Vidler,‡ Michael J. Watts,§ and John F. Hall‡

Cancer Biomarkers and Prevention Group, Biocenter, University of Leicester, University Road, Leicester, UK LE1 7RH, Cell Signaling Research Group, De Montfort University, Leicester, LE1 9BH, UK, and British Geological Survey, Kingsley Dunham Building, Keyworth, Nottinghamshire, NG12 5GG, UK

The production and evaluation of an isotopically enriched metalloprotein standard for use as a calibrant in species-specific isotope dilution analysis by HPLC coupled to inductively coupled plasma mass spectrometry is described. Using a model system involving the copper-containing protein rusticyanin (Rc) from the bacterium *Acido-thiobacillus ferrooxidans*, it was possible to demonstrate the analytical conditions that could be used for the measurement of metalloproteins by on-line IDMS analysis. Rc was chosen because it is a well-characterized protein with an established amino acid sequence and can be produced in suitable quantities using a bacterial recombinant system. Three different forms of the protein were studied by organic and inorganic mass spectrometry: the native form of the protein containing a natural isotopic profile for copper, an isotopically enriched species containing virtually all of its copper as the ^{65}Cu isotope, and the nonmetalated apo form. Incorporation of the copper isotopes into the apo form of the protein was determined using a UV−vis spectrophotometric assay and shown to be complete for each of the copper-containing species. The experimental conditions required to maintain the conformational form of the protein with a nonexchangeable copper center were established using +ve electrospray mass spectrometry. A pH 7.0 buffer was found to afford the most appropriate conditions, and this was then used with HPLC−ICP-MS to verify the stability of the copper center by analysis of mixtures of different isotopic solutions. No exchange of the enriched copper isotope from Rc with an added naturally abundant inorganic copper cation was observed under a neutral pH environment, indicating that species-specific ID-MS analysis of metalloproteins is possible.

On-line isotope dilution analysis using chromatography coupled to inorganic mass spectrometry (ICP-MS) has recently been developed into a useful approach to the quantification of elemental species in different materials[1] and to highlight systematic errors in some analytical elemental speciation methods.[2−4] This analytical approach offers a high degree of precision and accuracy, even at trace concentrations, and provides a means to verify speciation methods with a greater degree of confidence than methods using conventional analytical approaches.

A framework encompassing the different strategies for carrying out these measurements has been described:[5,6] species-specific spiking, whereby the sample is spiked with an internal standard containing an enriched isotope of the metal at the beginning of the analytical procedure and species-unspecific spiking in which the enriched spike is added continuously to the eluent from the chromatographic column. In both approaches, the isotope ratio between the spike and analyte isotope is measured via ICP-MS. The former method requires that the structure of the chemical species in the sample is known and that a suitable isotopically enriched spike material is available. The latter method has been used when the elemental species of interest is unidentified or a standard containing an enriched isotope is not available. Species-specific spiking is superior to the use of species-unspecific spiking because any chemical or physical losses of the analyte during the analytical procedure will be corrected for in the final ID-MS measurement, assuming that both the spike and the analyte reach chemical equilibrium prior to extraction. The real value of the species-specific approach was highlighted during the development of a GC/ICP-MS method for the analysis of methylmercury in different environmental water samples.[2] This work identified a systematic error during the derivatization step when employing ethylation, whereby the methylmercury in the sample was converted into elemental or inorganic mercury prior to analysis. However, the species-specific IDMS calibration method completely

* To whom correspondence should be addressed. Phone: +44 (0)116 223 1824. Fax: +44 (0)116 223 1840. E-mail: cfh7@le.ac.uk.

† University of Leicester.

‡ De Montfort University.

§ British Geological Survey.

(1) Clough, R.; Truscatt, J.; Belt, S. T.; Evans, E. H.; Fairman, B.; Catterick, T. *Appl. Spectrosc. Rev.* **2003**, *38*, 101−132.

(2) Demuth, N.; Heumann, K. G. *Anal. Chem.* **2001**, *73*, 4020−4027.

(3) Rahman, G. M. M.; Kingston, H. M. *Anal. Chem.* **2004**, *76*, 3548−3555.

(4) Monperrus, M.; Krupp, E.; Amouroux, D.; Donard, O. F. X.; Martin-Doimeadios, R. C. R. *Trends Anal. Chem.* **2004**, *23*, 261−272.

(5) Rottmann, L.; Heumann, K. G. *Fresenius' J. Anal. Chem.* **1994**, *350*, 221−227.

(6) Heumann, K. G.; Rottmann, L.; Vogl, J. *J. Anal. Atm. Spectrom.* **1994**, *9*, 1351−1355.

10.1021/ac050256t CCC: $30.25
Published on Web 05/10/2005

corrected for these transformations without any action being required and demonstrates one of the major advantages of using isotopically labeled calibrants in elemental speciation analysis.

The use of chromatography coupled to ICP-MS for the analysis of elemental speciation in biological samples has increased over recent years.[7] Numerous reports have detailed the analysis of organometallic compounds using this approach, and some of these have described the use of species-specific spiking with HPLC−ICP-MS to measure, for example, the chemical speciation of chromium,[8] lead,[9] mercury,[10] and selenium.[11] In contrast to the analysis of organometallic compounds, which have been separated by both gas and liquid chromatography, the analysis of metal-containing proteins has mostly been facilitated using size-exclusion chromatography (SEC) or capillary electrophoresis (CE) coupled to inorganic (ICP-MS) or organic (ESI-MS) mass spectrometry. A wide range of different metalloproteins have been determined using SEC−ICP-MS, including the cytosolic metal-binding proteins in polychaete worms,[12] the iron containing proteins in meat,[13,14] metallothioneins in fish kidney cytosols,[15] and a range of different commercially available metalloprotein standards.[16] CE−ICP-MS has been applied to the determination of metallothionein in commercially available standards;[17] the analysis of metallothionein in the liver tissues of rat,[18] fish, and deer;[19] and the metallothionein content of human brain cytosols.[20]

To date, only species-unspecific spiking has been applied to the determination of metalloproteins by on-line isotope dilution analysis.[15,20,21] A CE−ICP-(ID)MS instrumental setup was used for the quantification and stoichiometric evaluation (metal-to-sulfur ratios) of different isoforms of metallothionein in commercial rabbit liver preparations[21] and the metallothionein content of human brain cytosols.[20] Size-exclusion chromatography coupled to ICP-MS has been used in conjunction with species-unspecific quantitation of Cu, Zn, and Cd associated with metallothionein in carp and eel cytosols.[15] The ID-MS analysis of oxidized metallothionein extracted from rat liver carried out as a total analysis without a chromatographic or electrophoretic separation and employing a stable enriched inorganic cadmium spike has been reported.[22]

For the absolute quantitation of individual proteins in a complex mixture, methods based on using isotopically labeled protein standards are required to attain a sufficient degree of accuracy and precision. A number of methods for isotopically labeling proteins have been reported;[23,24] synthetically prepared proteins that either contain amino acids enriched with stable isotopes or are labeled by stable isotopes in a chemical or enzymatic reaction are commonly employed for this purpose. Proteins that are isotopically labeled have chemical properties almost identical to the native protein, but they can be distinguished because of the difference in masses between them. The aim of this present study was the production of a metalloprotein standard containing an enriched metal isotope for use as a spike material in species-specific isotope dilution analysis and therefore facilitation of the development of a new method for the absolute quantification of metalloproteins by HPLC−ICP-(ID)MS. ESI-MS was used to qualitatively evaluate the conformation of the protein under different solvent and pH environments and to determine the most appropriate chromatographic conditions for HPLC−ICP-MS analysis, where the native structure of the protein would be maintained and, hence, the copper isotope would be retained within the protein structure. The charge-state distributions formed during ESI-MS analysis can be used to characterize a wide range of protein structural features, providing compelling evidence as to the form of the protein under different experimental conditions.[25,26]

To determine the feasibility of using species-specific isotope dilution calibration of metalloproteins, we used the copper-containing protein rusticyanin (Rc) as a model system. This small (16 614 Da) monomeric type-1 blue copper protein was first isolated from *Acido-thiobacillus ferrooxidans.* Rc is thought to be a principal component in the respiratory electron transport chain of this Gram-negative bacterium and was chosen for this study because it is well-characterized, it has an established amino acid sequence,[27] and it can be produced in suitable quantities.[28] Production of Rc containing an enriched isotope of ^{65}Cu was facilitated using the cell culture and protein expression systems developed previously for spectroscopic and structural studies of the protein.[28] The isotopically enriched protein was fully characterized by standard biochemical assays, along with ESI-MS and HPLC−ICP-MS, to determine the stability of the metal center under different analytical conditions.

EXPERIMENTAL SECTION

Materials and Reagents. HPLC grade solvents were employed for this work, along with high-purity deionized water (>18 MΩ, Elga Ultrapure, UK). Other reagents included ammonium acetate (Analar, BDH, Dorset, UK), trifluoroacetic acid (Sigma-

(7) Szpunar, J.; Lobinski, R.; Prange, A. *Appl. Spectros.* **2003**, *57*, 102A−112A.
(8) Huo, D. W.; Kingston, H. M. *Anal. Chem.* **2000**, *72*, 5047−5054.
(9) Brown, A. A.; Ebdon, L.; Hill, S. J. *Anal. Chim. Acta* **1994**, *286*, 391−399.
(10) Clough, R.; Belt, S. T.; Evans, E. H.; Fairman, B.; Catterick, T. *J. Anal. At. Spectrom.* **2003**, *18*, 1039−1046.
(11) Ruiz Encinar, J.; Schaumloffel, D.; Ogra, Y.; Lobinski, R. *Anal. Chem.* **2004**, *76*, 6635−6642.
(12) Mason, A. Z.; Storms, S. D.; Jenkins, K. D. *Anal. Biochem.* **1990**, *186*, 187−201.
(13) Harrington, C. F.; Elahi, S.; Merson, S. A.; Ponnampalavanar, P. *Anal. Chem.* **2001**, *73*, 4422−4427.
(14) Harrington, C. F.; Elahi, S.; Merson, S. A.; Ponnampalavanar, P. *AOAC Int.* **2004**, *87*, 253−258.
(15) Goenaga Infante, H.; Van Campenhout, K.; Schaumloffel, D.; Blust, R.; Adams, F. C. *Analyst* **2003**, *128*, 651−657.
(16) Hann, S.; Kollensperger, G.; Obinger, C.; Furtmuller, P. G.; Stingeder, G. *J. Anal. Atm. Spectrom.* **2004**, *19*, 74−79.
(17) Mounicou, S.; Polec, K.; Chassaigne, H.; Potin-Gautier, M.; Lobinski, R. *J. Anal. Atm. Spectrom.* **2000**, *15*, 635−642.
(18) Polec-Pawlak, K.; Schaumloffel, D.; Szpunar, J.; Prange, A.; Lobinski, R. *J. Anal. Atm. Spectrom.* **2002**, *17*, 908−912.
(19) Profrock, D.; Prange, A.; Schaumloffel, D.; Ruck, W. *Spectrochim. Acta B* **2003**, *58*, 1403−1415.
(20) Prange, A.; Schaumloffel, D.; Bratter, P.; Richarz, A.-N.; Wolf, C. *Fresenius' J. Anal. Chem.* **2001**, *371*, 764−774.
(21) Schaumloffel, D; Prange, A.; Marx, G.; Heumann, K. G.; Bratter, P. *Anal. Bioanal. Chem.* **2002**, *372*, 155−163.
(22) Valles Mota, J. P.; Linde Arias, A. R.; Fernandez de la Campa, M. R.; Garcia Alonso, J. I.; Sanz-Medel, A. *Anal. Biochem.* **2000**, *282*, 194−199.
(23) Havlis, J.; Schevchenko, A. *Anal. Chem.* **2004**, *76*, 3029−3036.
(24) Barnidge, D. R.; Dratz, E. A.; Martin, T.; Bonilla, L.; Moran, L. B.; Lindall, A. *Anal. Chem.* **2003**, *75*, 445−451.
(25) Yan, X.; Watson, J.; Shing Ho, P.; Deinzer, M. L. *Mol. Cell. Proteomics* **2004**, *3*, 10−23.
(26) Dobo, A.; Kaltashov, I. A. *Anal. Chem.* **2001**, *73*, 4763−4773.
(27) Ronk, M.; Shively, J. E.; Shute, E. A.; Blake, R. C. *Biochemistry* **1991**, *30*, 9435−9442.
(28) Hall, J. F.; Kanbi, L. D.; Harvey, I.; Murphy, L. M.; Hasnain, S. S. *Biochemistry* **1998**, *37*, 11451−11458.

Aldrich, Dorset, UK), and tris(hydroxymethyl)aminomethane-hydrochloride pH 7.0 buffer (Sigma-Aldrich, Dorset, UK).

The macromolecular standards used to size-calibrate the gel filtration column included cytochrome *c* (12 kDa, bovine heart), myoglobin (16.9 kDa, horse skeletal muscle), and dextran blue (2000 kDa) (all Sigma-Aldrich, Dorset, UK). Stock solutions were dissolved in a pH 7.0 buffer and stored in amber glass bottles in a refrigerator prior to use.

Copper sulfate (Analar, BDH, Dorset, UK) with a natural isotopic abundance (%) of ^{63}Cu, 69.17% and ^{65}Cu, 30.83%[29] and a solution of copper metal (Spectrascan, Teknolab, Norway) with an enriched isotopic abundance (%) of ^{63}Cu, 0.39% and ^{65}Cu, 99.61%[30] were incorporated separately into the apo form of the protein as described below.

Preparation of the Metalloprotein Solutions. Rusticyanin was produced using cell culture and bacterial expression systems as reported in an earlier paper.[28] Briefly, the gene for Rc was cloned into pET21d and then used to transform *Escherichia coli* BL21 (DE3). Protein expression was then induced with isopropyl-β-D-thiogalactopyranoside (100 μg in 50 mL of fresh medium) after 200-mL cultures had been grown overnight. The cells were harvested by centrifugation and extracted with 5 M urea in 50 mM acetate pH 4.0. The extracts were then extensively dialyzed against the same buffer minus urea and centrifuged at 8000 rpm for 20 min to remove any precipitate. The supernatant was filtered through a 0.45-μm membrane prior to ion-exchange chromatography on a SP-Sepharose fast flow column. The extract was loaded in acetate buffer and eluted with a NaCl gradient (0.25−0.45 M) in acetate buffer. Due to the volume of the post dialysis, pre-cation exchange *E. coli* BL21 (DE3) cell lysate (~100 mL), it was necessary to perform the cation exchange purification in three batches, that is, ~30 mL of lysate was loaded onto the column per purification. Rc produced in the same expression experiment was used for all three Rc solutions detailed below. Fractions containing the expressed protein were collected and stored at 4 °C until required.

Prior to addition of the copper isotope to the apo-protein, the approximate protein concentration was determined using the Bradford assay.[31] The partially purified apo-protein solution was then split into two aliquots. The pH of the naturally abundant aqueous copper sulfate solution was measured at about pH 4. The enriched inorganic copper solution in 2.5% nitric acid was adjusted to match this pH using dilute ammonia solution, dilute hydrochloric acid solution, and dilute sodium hydroxide solution, prior to its addition to the apo-protein. This pH adjustment was necessary to reduce the protein precipitation observed when using a lower pH. A 5-fold molar excess of copper, as compared to the protein, was used to facilitate incorporation of the copper into the apo-protein. After ~30 min, both copper-containing protein solutions had developed a slightly blue color, characteristic of the metalated protein form. Excess copper was removed from solution using centrifugal filters with a 5000 nominal molecular weight limit (NMWL) (Centricon Plus-20 Biomax-5, Millipore). The apo-Rc solution was prepared in a similar way, but without the addition of copper, using a different purification of the same protein expression experiment.

Protein Characterization. The reagents used for sample preparation and MS analysis were analyzed for total copper content using an accredited ICP-MS method (UK Accreditation Service). The extent of copper incorporation into the protein was established using a UV−vis spectrophotometric assay[32] as follows. Each solution (2.5 mL) was fully oxidized by addition of 20 μL of 50 mM aqueous potassium hexachloroiridate (Aldrich, WI) to convert the copper center to the Cu(II) oxidation state. After standing at room temperature for 60 min, each solution was purified using a PD-10 desalting column (Amersham Biosciences, Buckinghamshire, UK) to remove the brown potassium hexachloroiridate to prevent it from interfering in the following measurement. The absorbance of both solutions was measured at 280 and 597 nm using a benchtop UV−vis spectrophotometer (Unicam model 5625, UK), and a ratio of the values was determined.

Characterization of the Protein by Electrospray Mass Spectrometry. Each of the three forms of rusticyanin (apo, natural Cu, and enriched Cu) were structurally characterized using +ve electrospray mass spectrometry (ESI-MS) (Micromass Q-TOF Ultima, Waters Ltd, Manchester, UK). Prior to the ESI-MS analysis, the protein solutions were extensively desalted (three separate dilution/concentration steps) with molecular weight cutoff filters (YM-10, Microcon, Millipore) using the protocol provided by the manufacturer. The purified protein solutions were diluted (1 + 9) to a final concentration of ~5 pmol μL^{-1}, with solutions of different composition, to provide a range of denaturing or nondenaturing conditions. Tris(hydroxymethyl)aminomethane-hydrochloride pH 7.0 buffer (50 mM), ammonium acetate pH 6.9 (50 mM), and acetonitrile/water (1:1 v/v) with trifluoroacetic acid (1% v/v) were each evaluated.

Electrospray mass spectra were obtained by infusing the sample into the source at a few microliters per minute using a syringe pump (Harvard Apparatus, South Natick, MA) and the instrumental parameters given in Table 1. Different mass spectrometer parameters were required for the denaturing and the nondenaturing experiments, which were optimized using a myoglobin standard (3 pmol μL^{-1}) prepared in the appropriate solution. CsI was used to calibrate (m/z) the instrument, and the accuracy of the calibration was confirmed using a myoglobin standard. The raw spectra were smoothed using the manufacturer-supplied Savitzky−Golay algorithm prior to spectral deconvolution using the MaxEnt1 program supplied with the instrumental software.

Evaluation of the Isotopically Enriched Metalloprotein Stability. A Progel (TSK Gel G2000$_{SW}$, Supelco, Dorset, UK) gel filtration column (GFC), 300 × 7.6 mm i.d., 10 μm, 125 Å, with a GFC-2000 guard cartridge (Phenomenex, Maccelsfield, UK), was coupled to the nebulizer of an ICP-MS instrument (ExCell, VG Instruments, Manchester) via a short piece of PEEK tubing. Sample loading was achieved using a metal-free injection valve (model 9125, Rheodyne, Rohnert Park, CA) equipped with a PEEK 100-μL sample loop (Alltech, Carnforth, UK). The mobile phase contained 50 mM tris(hydroxymethyl)aminomethanehydrochloride pH 7.0 buffer and was pumped (HP 1050 isocratic pump) at a flow rate of 0.5 mL min^{-1}, producing a column head pressure of 23

(29) Horlick, G.; Montaser, A. In *Inductively Coupled Plasma Mass Spectrometry*; Montaser, A., Ed; Wiley-VCH: New York, 1998; pp 503−612.
(30) Teknolab, Norway. *Certificate of analysis*, article no. 50010, Lot no. 1; 2003.
(31) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248−254.
(32) Casimiro, D. R.; Toy-Palmer, A.; Blake, R. C.; Dyson, H. J. *Biochemistry* **1995**, *34*, 6640−6648.

Table 1. Instrumental Parameters Used for the ESI-MS and ICP-MS Experiments

ESI-MS Parameters	
instrument	Micromass Q-TOF Ultima
capillary voltage	+3.50 V
cone voltage	70 V
source temperature	70 °C
desolvation temperature	120 °C
desolvation gas flow	350 L h^{-1}
collision cell	argon at 1 bar
scan range	500−3000 m/z
acquisition	scan time, 1 s; interscan delay, 0.1 s
ICP-MS Parameters	
instrument	VG Instruments Excell
nebulizer	Micromist Lo-flow (Glass Expansion, Switzerland)
spray chamber	glass impact bead peltier cooled to 4 °C
gas flow rates	coolant, 13; auxiliary, 0.94; nebulizer, 0.94 (L min^{-1})
RF power	1.3 kW
torch	standard demountable quartz torch with 1.5-mm-i.d. injector
cones	nickel sampler and skimmer
collision cell gas flow rates	helium, 2.0; hydrogen, 1.0 (mL min^{-1})
isotopes monitored	^{63}Cu, ^{65}Cu, ^{52}Cr, ^{53}Cr, ^{56}Fe, ^{64}Zn, ^{65}Zn, ^{66}Zn, ^{77}Se, ^{82}Se, ^{115}In
dwell times	Cu isotopes, 10 ms; all others, 40 ms

bar. The buffer was prepared by mixing 200 mL of 250 mM Tris base with 190 mL of 250 mM HCl, followed by dilution to 1 L with DI water. The GFC column was size-calibrated using different metal-containing macromolecular standards (1000 mg L^{-1} as compound) made up in the mobile phase. To monitor the stability of the plasma during the chromatographic run, indium (10 μg L^{-1} in 1% nitric acid) was added postcolumn via a tee-piece at a flow rate of 0.8 mL min^{-1}. HPLC−ICP-MS analysis was performed using the instrumental parameters in Table 1 by analyzing the two copper isotopes at m/z 63 (natural Cu isotope) and m/z 65 (spike Cu isotope) using the time-resolved acquisition (TRA) software program.

RESULTS AND DISCUSSION

Determination of Copper Incorporation and Total Copper Concentration. Only trace amounts (low nanograms per gram) of copper were determined in the reagents used to prepare the protein, and the apo-protein solution contained only minor amounts of copper prior to metalation. The extent of incorporation of the naturally abundant Cu and enriched Cu isotopes into the apo-Rc were evaluated using the spectrophotometric method established by Casimiro et al.[32] The protocol involves oxidation of the copper center in the protein to the Cu(II) oxidation state and measurement of absorption at 280 and 597 nm. An absorption ratio (A_{280}/A_{597}) of 7 or less indicates 100% incorporation of copper into the apo-protein. The ratios determined in this way are shown in Table 2. The Bradford assay[31] was used to provide an estimate of the total protein concentration of the three solutions. The values determined for each form of Rc are given in Table 2. The values shown in Table 2 indicate that the total protein solution concentrations are similar in each copper Rc solution and that the copper isotopes from the two different isotopic solutions have been fully incorporated into the apo-protein solutions.

Table 2. Typical UV-Vis Absorbance Data Illustrating Copper Incorporation in the Three Forms of Rc and Their Approximate Concentration as Determined Using the Bradford Assay

	absorbance			
Rc protein soln	280 nm	597 nm	A_{280}/A_{597}	concn, mg L^{-1}
apo-Rc	0.308	0.002	154	1221
natural Cu−Rc	0.303	0.043	7.05	774
enriched Cu−Rc	0.200	0.029	6.90	817

Characterization of the Protein by +ve ESI-MS. The approach used to study the metal-binding characteristics of Rc and the stability of the metal center under different analytical conditions was based on previous work using ESI-MS to characterize metallothionein.[33−35] These reports showed that by altering the pH of the metallothionein isoforms under investigation, either before continuous infusion[33] or postcolumn when using HPLC coupled to ESI-MS,[34,35] it was possible to determine the stoichiometry (metal-to-protein ratio) of the metalloprotein with improved detection limits, in a single analyses.

The results for the two different Rc metalated protein solutions evaluated using this type of approach are shown in Figure 1, and the deconvoluted spectra obtained using the MaxEnt1 software are also included for each protein. When tested under nondenaturing conditions using Tris pH 7.0 buffer (Figure 1a) or ammonium acetate pH 6.9, five charge states were observed (+10 to +6) in the mass range studied. The molecular weight determined for each protein solution was in agreement with the value calculated from the published amino acid sequence (16 614 Da) and indicates that the protein structure under these conditions is such that the copper atom is still present using both of the neutral pH conditions tested. The results shown in the deconvoluted spectra (Figure 1a and 1b) indicate the presence of a small amount of apo-Rc (16 551 Da) due to the loss of a copper atom (∼10−15% of the total protein signal at 16 614 Da). The neutral buffer conditions required to maintain a stable protein structure and the requirement to obtain a reasonable ion signal necessitated desolvating parameters where the loss of the copper atom was more likely to occur.[25,36] The most probable explanation for the loss of the Cu atom is that it results from reactions occurring in the gas phase during the electrospray ionization process and does not reflect the situation when the protein is in solution.

The mass spectra and deconvoluted spectra for the enriched metal-containing protein made up in a solution containing acetonitrile/water (1:1 v/v) and trifluoroacetic acid (1%) are shown for comparison in Figure 1c. In this case, the protein is effectively denatured when an organic modifier and TFA are present, leading to loss of the Cu atom and the formation of a greater number of charge states (+19 to +8) centered on a lower m/z range. This mass spectra is markedly different from the two previous ones obtained for the nondenaturing conditions because it contains a much greater number of charged states, which result from the presence of the organic modifier and the low pH, which effectively

(33) Yu, X.; Wojciechowski, M.; Fenselau, C. *Anal. Chem.* **1993**, *65*, 1355−1359.
(34) Chassaigne, H.; Lobinski, R. *J. Chromatogr., A* **1998**, *829*, 127−136.
(35) Le Blanc, J. C. Y. *J. Anal. Atm. Spectrom.* **1997**, *12*, 525−530.
(36) Loo, J. A. *Mass Spectrom. Rev.* **1997**, *16*, 1−23.

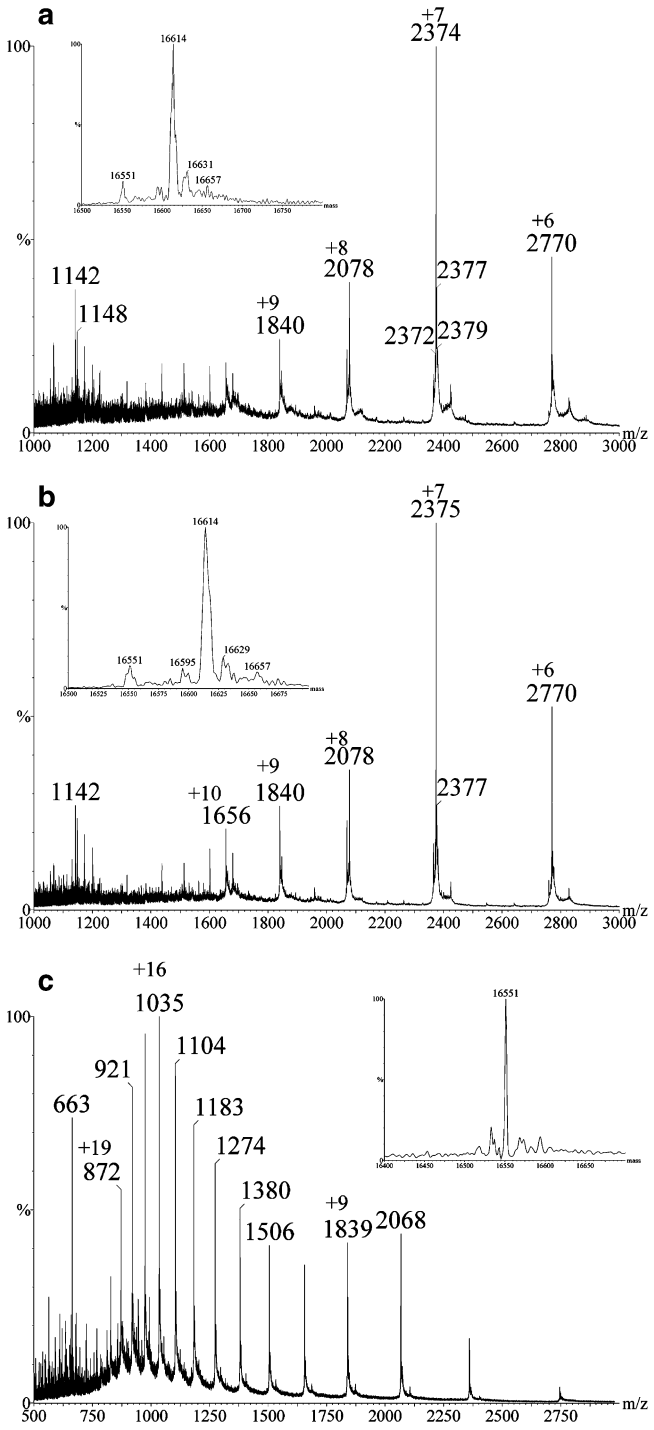

Figure 1. (a) +ve ESI-MS analysis of natural Cu−Rc made up in Tris (50 mM) pH 7.0 buffer. Inset shows the deconvoluted spectra obtained using the MaxEnt1 software. (b) +ve ESI-MS analysis of enriched Cu−Rc made up in Tris (50 mM) pH 7.0 buffer. Inset shows the deconvoluted spectra obtained using the MaxEnt1 software. (c) +ve ESI-MS analysis of enriched Cu−Rc made up in acetonitrile/water (1:1 v/v) containing trifluoroacetic acid (1%). Inset shows the deconvoluted spectra obtained using the MaxEnt1 software.

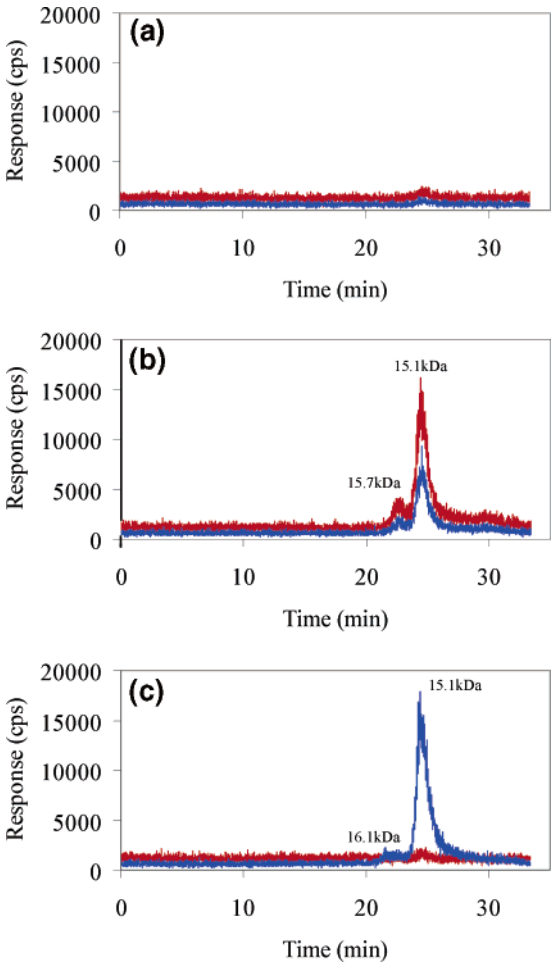

Figure 2. (a) HPLC−ICP-MS chromatogram for a solution of apo-Rc: blue, ^{65}Cu; red, ^{63}Cu. (b) HPLC−ICP-MS chromatogram for a solution of natural Cu−Rc: blue, ^{65}Cu; red, ^{63}Cu. (c) HPLC−ICP-MS chromatogram for a solution of enriched Cu−Rc: blue, ^{65}Cu; red, ^{63}Cu.

open up the protein and facilitate the accessibility of more sites that can be charged. No real consistency in the mass spectra for each separate compound were obtained at different times and using different tune parameters, either for Rc or the myoglobin standard used to tune the instrument; however, there was a high degree of consistency in the deconvoluted spectra generated over time. The only consistent features were that the spectra obtained under denaturing conditions contained a much higher number of charge states, as compared to those at neutral pH conditions. These results correspond to other studies that have investigated the noncovalent binding properties of proteins,[36] particularly metalloproteinase enzymes and previous reports on the structure of Rc using electrospray mass spectrometry under acidic conditions.[27,28]

Characterization of the Protein by HPLC−ICP-MS. The results from the analysis of the Rc solutions using the size-calibrated gel filtration column (GFC) are shown in Figure 2. A minor peak on both copper isotope channels is present in the chromatogram for the apo-Rc solution (Figure 2a), which could result from the minor copper content of the apo-Rc solution, because no effect on the plasma was noted on the indium channel (m/z 115) during elution of the protein. Two peaks are present in the HPLC−ICP-MS chromatogram (Figure 2b) for Rc containing the naturally abundant Cu. The first minor peak equates to a protein of mass 15.7 kDa; the second major peak in the chromatogram has a nominal mass of 15.1 kDa. The nominal mass of native Rc containing a copper atom is 16.6 kDa, which is different from both of the peaks observed in this experiment. This difference in observed mass and the literature value is not

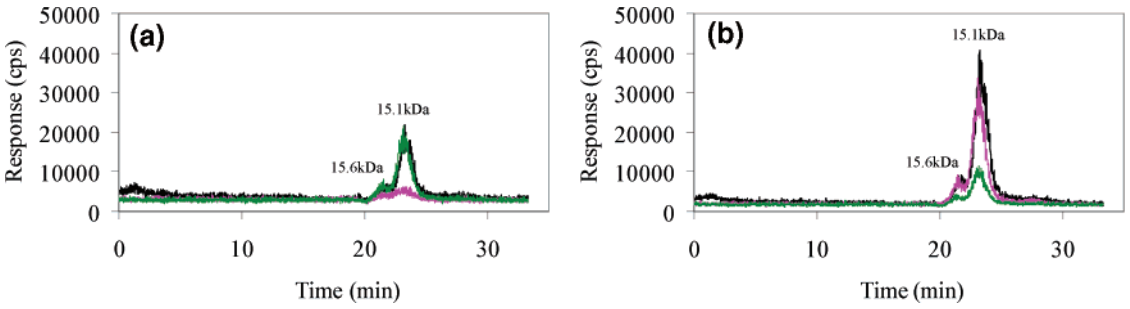

Figure 3. (a) Comparison of the ^{63}Cu isotopic signal for mixtures (1:1 v/v) of natural Rc and apo-Rc (green), enriched Rc and apo-Rc (pink), and enriched Rc and natural Rc (black) generated by HPLC−ICP-MS analysis. (b) Comparison of the ^{65}Cu isotopic signal for mixtures (1:1 v/v) of natural Rc and apo-Rc (green), enriched Rc and apo-Rc (pink), and enriched Rc and natural Rc (black) generated by HPLC−ICP-MS analysis.

uncommon when using GFC to estimate the size of proteins and has been observed previously when using columns of this type.[13,37] The chromatogram (Figure 2c) for Rc containing the enriched isotope of copper contains one major and a very minor peak on the ^{65}Cu channel and no peaks present on the ^{63}Cu channel. The peaks correspond to proteins of mass 15.1 kDa (major) and 16.1 kDa (minor), respectively. After further sample analysis, a slightly larger first peak with a nominal mass of 15.7 kDa was observed in some samples. It is therefore apparent from this study that Rc elutes as two peaks, a minor peak with a nominal mass of 15.7 kDa and a major peak of mass 15.1 kDa, on the GFC column used in this work. As noted above, the behavior of some proteins on silica-based columns, such as that used in this work, is nonideal in nature and can lead to nonsymmetrical or split peaks.[38] However, because this type of column is the most commonly used in studies of metalloproteins with ICP-MS detection, its characteristics were evaluated using the enriched Rc metalloprotein, rather than using a polymeric-based alternative.

Stability of the Protein under IDMS Conditions. The main consideration when evaluating the potential for using recombinant proteins containing isotopically enriched metal atoms as a spike solution for species-specific IDMS analysis is that the isotopic abundance of the metal center being measured does not change during the analytical procedure. In practice, it is essential that the enriched isotope in the spike protein, once equilibrated with the native protein being measured, is not lost from the protein, generating a site that could take up any natural inorganic copper present in solution. Unlike species-specific IDMS of organometallic cations, such as tributyltin or methymercury, in which the metal is covalently bound to the ligands present and any change in molecular structure can be accounted for by the IDMS process,[2] the loss of the enriched metal from the protein in the presence of inorganic copper could lead to a change in the equilibrium between the spike and analyte isotopes in the different protein solutions. IDMS would not correct for this even if the analyte protein being measured also lost copper in the same way (which would be the case, because there is no difference in chemical structure or activity between the two proteins). Both proteins would have the potential to regain a copper atom, and if there were an excess of inorganic natural copper in the solution being measured (from reagents or other sources), the isotopic content of the enriched protein would be altered. In this way, some of the spike material could be converted to the natural Cu form. To test this hypothesis, we mixed the different protein solutions together and also with inorganic copper with a different isotopic abundance.

Both metalloprotein solutions were spiked (1:1 v/v) with an aliquot of the apo-protein solution, and each metalloprotein was mixed together (1:1 v/v) to determine if this had any effect on the elution characteristics or isotopic content of the natural and enriched metal protein solutions. The results presented in Figure 3a show the effect on the ^{63}Cu profile of preparing binary solutions of the three different proteins. The enriched Cu−Rc protein contains >99% of its copper as ^{65}Cu, so on mixing with the apo-Rc, the peak for ^{63}Cu at the elution point for Rc is very small, and the signal that is present could be due to residual sodium associated with the protein, which can form a polyatomic interference at the m/z of interest due to the formation of $^{40}Ar^{23}Na$ (99.6%). The chromatograms for the mixtures containing natural Cu−Rc with either apo-Rc or enriched Cu−Rc are essentially equivalent in terms of peak height or area, as expected, because there is no ^{63}Cu contribution to the signal from either of the other protein solutions. The analogous results for the ^{65}Cu signal for each of the mixtures described are shown in Figure 3b, and in this case, there is a contribution to the ^{65}Cu peak from both of the metalated forms of Rc. It is evident from this chromatogram that the peak profile for the mixture of the natural Cu−Rc and the enriched Cu−Rc is essentially a summation of the other two profiles, as expected. These two figures indicate that the use of species-specific IDMS of metalloproteins may be possible from a chromatographic perspective because all the proteins behave as expected when mixed. However, it does not provide any evidence that redistribution of the copper center does not occur in this system.

The Potential for Using Isotopically Enriched Metalloproteins for On-Line IDMS. By spiking the enriched Cu−Rc protein solution with an aliquot of the natural inorganic Cu isotope, it was possible to evaluate the stability of the metal center and establish whether copper redistribution occurs under the neutral pH chromatographic conditions employed during this experimental study. Figure 4 a shows the results for the analysis of the enriched Cu−Rc protein solution spiked (1:1 v/v) with an inorganic solution of naturally abundant copper. This chromatogram shows that the ^{65}Cu atom in the protein does not exchange with the inorganic ^{63}Cu that was added to the solution and that the copper center is stable during the chromatographic separation. The copper isotopic

(37) Oellingrath, I. M.; Slinde, E. *J. Food Sci.* **1985**, *50*, 1551−1555.
(38) Kopaciewicz, W.; Regnier, F. E. *Anal. Biochem.* **1982**, *126*, 8−16.

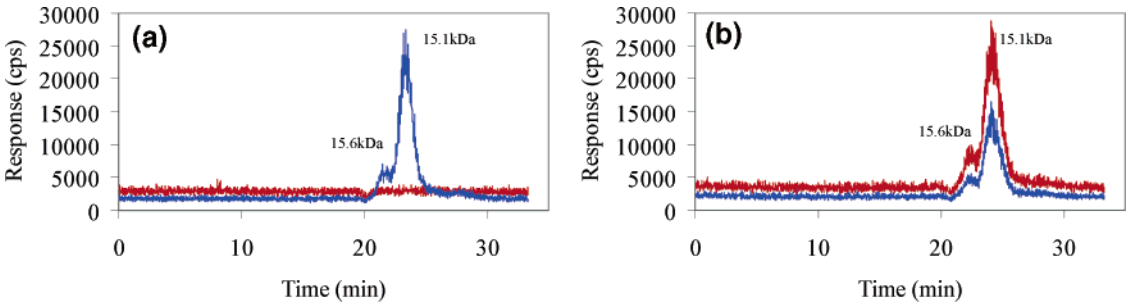

Figure 4. (a) HPLC−ICP-MS chromatogram for a mixture (1:1 v/v) of enriched Rc and natural inorganic copper: blue, ^{65}Cu; red, ^{63}Cu. (b) HPLC−ICP-MS chromatogram for a mixture (1:1 v/v) of apo-Rc and natural inorganic copper: blue, ^{65}Cu; red, ^{63}Cu.

composition of the enriched protein was unchanged when compared to an unspiked solution (see Figure 2c), and it is clear that there is no peak on the ^{63}Cu channel at the elution point for Rc. A similar approach was attempted for the natural Cu−Rc using a solution of the enriched inorganic copper, but because of problems related to the composition of the enriched isotopic solution, it was not possible to obtain a suitable sample for analysis. The apo-Rc form was spiked (1:1 v/v) with a solution of natural inorganic copper to determine its potential to accommodate the inorganic copper atom. The results are shown in Figure 4b. In comparison to Figure 1a, it is clear from this chromatogram that the apo form of the protein can take up any available copper that is present in the solution being analyzed. This is as expected and indicates that to use isotopically enriched recombinant proteins as spikes for species-specific IDMS, it must be established that the protein solutions to be used, including the analyte form being measured, are completely saturated with the metal that will be measured prior to analysis and that the conditions used must not lead to exchange or loss of the metal center. The model system evaluated in the current study did not involve a "real" sample matrix, which could contain other Cu-complexing ligands with the potential to interfere with the metal center in the analyte and spike, altering their isotopic equilibrium. However, by carrying out very controlled experiments, it may be possible to measure different proteins with high accuracy by the analysis of the metal center present, once the stability of the protein or its metal binding constant has been established under the experimental conditions being used.

The species-unspecific calibration method mentioned earlier allows for the quantification of the metalloprotein and also the determination of the stoichiometric composition of the metal complex understudy. Assuming that the amino acid composition of the protein is known or can be determined by ESI-MS/MS, by measuring the isotope ratios of the trace metals of interest and also of the covalently bound sulfur present in the protein itself, the number of bound metal atoms per molecule of protein can be determined. In contrast to this, the current method requires extensive knowledge of the protein, along with considerable expertise in biochemical techniques, to be able to generate the recombinant protein standard necessary for species-specific IDMS. However, it does have the advantage over the former method in that it is applicable to metalloproteins, such as Rc, that only have a small number of sulfur-containing amino acids present (2.5%, as compared to 30% in metallothionein). The measurement of sulfur isotope ratios requires the use of a high-resolution instrument or a collision/reaction cell instrument to eliminate the polyatomic interferences from oxygen. The method described here would therefore be applicable to a wider range of metalloproteins.

The use of species-specific spiking can improve the determination of specific biochemical species, such as metalloproteins, in a number of ways. By generating recombinant proteins, an exact analytical standard can be produced for the protein of interest. This greatly enhances the traceability of the analytical procedure and improves the reliability of the result. Currently, few metalloprotein standards are commercially available, which means that the analyst must rely on using proteins from one animal species to determine the concentration of the protein in another species. This is a far from adequate scenario because there may be subtle differences in the amino acid sequence between the proteins, which could affect the binding and behavior of the metalated form. The use of isotopically enriched metalloproteins also provides a means to evaluate the analytical conditions under which the metal center is most stable. Because the majority of metals within metalloproteins are held in place by noncovalent interactions, structural changes caused by changes in the chemical environment (such as encountered during the sample extraction process) would be expected to alter the protein/metal ratio by facilitating loss of the metal. When using inorganic mass spectrometry in combination with recombinant proteins containing an enriched metal isotope, the stability of the analyte can be established under both the extraction and analysis conditions. Currently, this is difficult to do because of the lack of standards for the different isoforms of the metalloproteins studied to date, as well as the complete lack of certified reference materials in this area.

CONCLUSIONS

To our knowledge, this is the first report showing that it is possible to generate metalloproteins containing an enriched metal isotope for use in species-specific ID-MS. The study also shows that the metal center was stable under the HPLC−ICP-MS instrumental conditions evaluated and that this approach could therefore form the basis of a high-accuracy method for the analysis of metalloproteins.

ACKNOWLEDGMENT

The authors acknowledge the UK Medical Research Council for support under Grant No. G0100873 and the British Geological Survey for the provision of ICP-MS instrument time. D.S.V. acknowledges the award of an EPSRC CASE studentship in collaboration with LGC (Teddington) Ltd, which was funded by

the UK Governments, Department of Trade and Industry as part of the VAM program. Thanks to J. H. Lamb and D. J. L. Jones for technical assistance with the ESI-MS measurements.

Note after ASAP Posting. This paper was inadvertently posted on the Web on May 10, 2005, before all corrections had been made. Corrections to the caption of Figure 2 and text changes to the third paragraph of Results and Discussion were made, and the paper was reposted on May 16, 2005.

Received for review February 10, 2005. Accepted April 8, 2005.

AC050256T