

Characterization of Metalloproteins Using Capillary Electrophoresis

Mark P. Richards

Growth Biology Laboratory, USDA-ARS-ANRI, Beltsville, Maryland, U.S.A.

Introduction

Metalloproteins constitute a distinct subclass of proteins that are characterized by the presence of single or multiple metal ions bound to the protein by interactions with nitrogen, sulfur, or oxygen atoms of available amino acid residues or are complexed by prosthetic groups, such as heme, that are covalently linked to the protein. These metals function either as catalysts for chemical reactions or as stabilizers of the protein tertiary structure. Protein-bound metals may also be labile and, as such, be subject to transport, transient storage, and donation to other molecular sites of requirement within tissues and cells.

Background Information

Metalloproteins play critical roles in a wide variety of basic cellular functions, including respiration, gene expression, reproduction, and metabolism. Isolation, characterization, and quantification of individual metalloproteins are each necessary and important steps toward understanding their unique biological functions. Alone or in combination, various types of chromatography, electrophoresis, and spectrometric techniques have been employed to study many unique aspects of metalloprotein structure and function. However, no one technique currently offers the ability to isolate, characterize, and quantify individual metalloproteins in a single step from complex matrices such as tissue extracts or physiological fluids. Therefore, there is an ongoing need for new and more capable methodologies. Because of the small-sample volume requirement, high degree of resolution, and advanced instrument automation capabilities, capillary electrophoresis (CE) has gained increasing popularity in the analysis of proteins [1,2]. In fact, many of the CE-based techniques developed for general protein separations are directly applicable to metalloprotein analyses [2,3]. This article will emphasize some recent applications of CE and CE-related methodologies and their utility in providing new insight into the structure and function of a variety of metalloproteins.

Table 1 summarizes some of the ways CE has been applied to metalloprotein characterization. Altering capillary temperature, buffer ionic strength and pH, electric field strength (i.e., voltage), and capillary internal surface coating are but a few of the ways that CE conditions can be varied to influence the efficiency and selectivity of metalloprotein separations. Furthermore, different CE separation modes can be applied to gain new information about a specific metalloprotein [3]. For instance, capillary zone electrophoresis (CZE) can indicate a protein's net charge at a given pH; capillary isoelectric focusing (CIEF) gives a rapid estimate of its isoelectric point (pI); capillary gel electrophoresis (CGE), in the presence of sodium dodecyl sulfate (SDS), can be used to estimate its apparent molecular mass; and micellar electrokinetic capillary chromatography (MEKC) can be useful in characterizing its surface hydrophobicity.

Procedures

Varying CE separation conditions has been shown to be a particularly effective approach for improving the isolation and characterization of metallothioneins, a heterogeneous family of low-molecular-weight, cysteine-rich, heavy-metal-binding proteins [4]. It was found that (a) phosphate and borate buffers enhanced the sensitivity of detection at 200 nm by significantly reducing ultraviolet (UV) absorption of the background electrolyte, (b) the alkaline borate buffer (pH 8.4) gave rapid analysis times with reasonably high resolution, (c) the acidic phosphate buffer (pH 2.5) completely stripped zinc and cadmium from the proteins, yielding higher resolution and more reproducible separations of the apothioneins (metal-free proteins), (d) capillaries coated on their inner surface with a polyamine polymer that reversed electro-osmotic flow (EOF) or polyacrylamide that greatly suppressed EOF significantly improved resolution, (e) the MEKC mode of CE improved separation selectivity, and (f) photodiode array scanning detection to monitor UV absorption spectra of individual protein peaks separated at

Table 1 Characterization of Metalloproteins Using Capillary Electrophoresis

Identification and Purity Assessments
General characteristics (i.e., net charge, molecular weight, isoelectric point, etc.)
Monitoring purification steps
Separation of impurities or degradation products
Detection of unique UV/visible absorbance (chromophore)
Structural Information
Separation of molecular forms (i.e., isoforms, metalloforms, glycoforms, etc.)
Study of macromolecular assembly
Determination of metal-binding sites
Peptide mapping
Stability Determinations
Effects of temperature and pH
Buffer additives (i.e., metals and metal chelators)
Activity Measurements
Enzymatic activity
Electrophoretically mediated microassay (EMMA)
Isozyme profiling
Metal-Binding/Electrophoretic Mobility Shift
Affinity CE (ACE)
Immobilized metal-ion affinity CE (IMACE)
Metal-chelate coated capillaries
<i>REDOX</i>
oxidation state of protein-bound metal
Elemental Analyses
Indirect Detection Methods
Unique UV/visible absorbance spectra (chromophore)
Mass spectrometry (CE-MS)
Direct (Element-Specific) Detection Methods
Inductively coupled plasma-mass spectrometry (CE-ICP-MS)
Proton-induced x-ray emission (PIXE)

neutral pH was useful in determining both the presence and the type of metal associated with each.

Capillary electrophoresis is a useful tool for monitoring the purity of metalloproteins isolated from either natural or recombinant sources [3]. CZE was used to follow the purification progress of metallothioneins in samples subjected to gel filtration chromatography and reversed-phase high-performance liquid chromatography (HPLC) [3,4]. Detection of a unique chromophore arising from the interaction of metal ions and specific amino acid residues in the protein or with a prosthetic group attached to the protein can be useful. The selectivity achieved under such conditions can greatly reduce or even eliminate the need to purify metalloproteins prior to their analysis by CE. Two good examples of this are the detection of hemoglobin variants separated from red blood cell lysates by monitoring absorbance at 415 nm and the detection of transferrin in serum at 460 nm [3]. Absorbance at these characteristic wavelengths reflects the presence

of iron atoms complexed by the heme moiety (hemoglobin) or by iron-binding sites located at the amino and carboxyl ends of the transferrin protein molecule. CE has also been used to characterize surface metal-binding sites on cytochrome-*c* and myoglobin modified with ruthenium-bis(bipyridine)imidazole, which imparts a strong absorbance at 292 nm to the modified proteins or peptides derived from a tryptic digest of the modified proteins [3].

Capillary electrophoresis has proven to be useful in characterizing different molecular forms of various metalloproteins like metallothionein, transferrin, and conalbumin [2–5]. Molecular forms arise from differences in the amino acid sequence of proteins (isoforms), differences in the amount or type of metal bound (metalloforms), or from differences in the type and amount of carbohydrate side chains linked to the protein (glycoforms). CZE was used to follow the formation of the oligomeric iron core and its incorporation into ferritin, to detect and quantify ferritin species

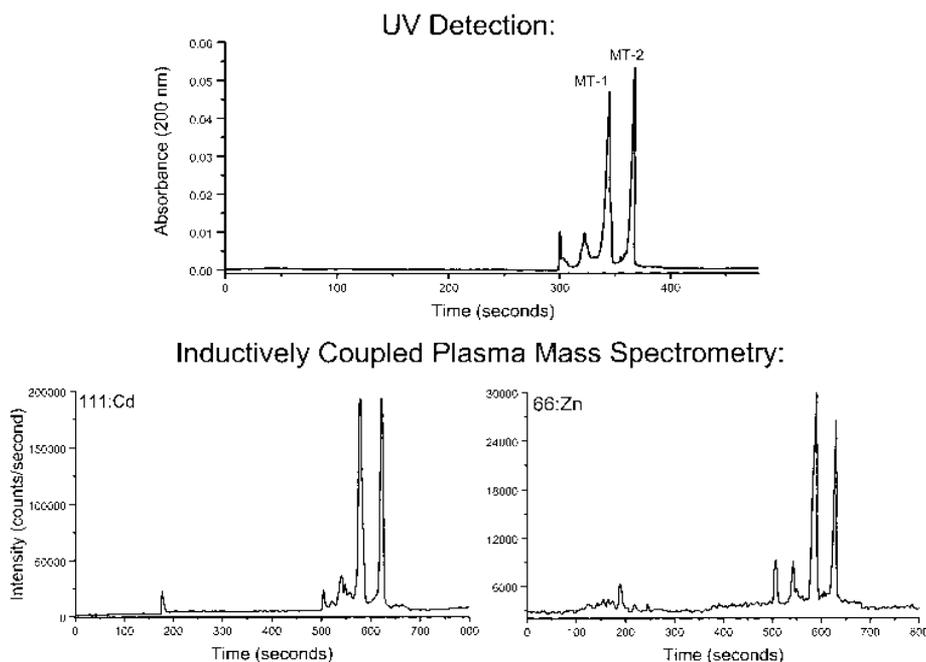


Fig. 1 Separation of rabbit liver metallothionein using CE-ICP-MS. The protein sample (1 mg/mL dissolved in deionized water) was first subjected to CZE with UV detection to optimize CE separation parameters for the major metallothionein isoforms (MT-1 and MT-2) shown in the upper panel. The CE instrument was then coupled to an ICP-MS instrument using a specially modified direct injection nebulizer (CETAC Technologies Inc., Omaha, NB) which enabled the entire capillary effluent from the CE to be directly injected into the ICP plasma torch, thus avoiding postcolumn dilution and band-broadening effects of conventional spray chamber nebulizers. Specific isotopes of cadmium (^{111}Cd) and zinc (^{66}Zn) associated with each isoform peak were monitored as shown by the figures in the lower panel.

or ferritin subunit proteins in purified or partially purified states, and to study the interaction of different metal ions with ferritin [2,3].

Structural stability of metalloproteins can be quickly assessed by CE under different conditions [2,3]. For example, thermally induced conformational changes in calcium-depleted α -lactalbumin and urea-induced unfolding of serum albumin were studied using CZE. The oxidation state of cysteine sulfhydryl groups in the zinc-containing protein, ribonuclease A, has been assessed using CZE to determine the presence or absence of a disulfide bond. Elevated capillary temperature altered the structure of myoglobin, which, in turn, resulted in reduction of the valence state of the iron atom bound to heme associated with this protein. Similarly, CIEF was used to separate and characterize different heme-iron valence hybrids of hemoglobin [6].

Buffer additives, especially metals and metal chelators, can have dramatic effects on CE-based separations of metalloproteins by causing shifts in their electrophoretic mobility [3]. This observation forms the

basis for a unique CE method referred to as affinity capillary electrophoresis or ACE. When a protein forms a complex with a charged metal-ion ligand, there can be a resulting change in electrophoretic mobility of the complexed protein relative to that of the metal-free protein. Scatchard analysis of the change in electrophoretic mobility of the protein as a function of the metal ion concentration in the separation buffer allows for the calculation of a metal-binding constant (K_b). ACE has been used to characterize K_b values for several metalloproteins, including (a) calcium affinity for calmodulin and C-reactive protein [3] and (b) the binding affinity of zinc for two separate sites in a highly basic, zinc-finger protein (NCp7) from the human immunodeficiency virus [7]. Haupt et al. [8] reported the development of an alternative CE affinity method based on immobilized metal affinity chromatography, which they called immobilized metal-ion affinity capillary electrophoresis or IMACE. In IMACE, metal ions (e.g., Cu^{2+}) are fixed to a soluble polyethylene glycol replaceable polymer matrix support added to the CE separation buffer. IMACE was used to study sur-

face-related affinity characteristics (number and accessibility of histidine residues and histidine microenvironment) for particular immobilized metal-ion chelate ligands in such proteins as cytochrome-*c*, ribonucleases A and B, chymotrypsin, and kallikrein [8].

Some of the most promising advances in our understanding of unique characteristics of metalloprotein structure and function come from continuing developments in detection methodologies and from further development and refinement of coupled (hyphenated) systems such as CE–mass spectrometry (CE–MS) and CE–inductively coupled plasma–mass spectrometry (CE–ICP–MS). The major difficulties restraining the routine use of such systems, aside from cost, arise from problems in interfacing the CE instrument with MS and ICP–MS instrumentation, although much progress is being made in this area [9]. CE–MS has been used to characterize metallothionein isoforms and metalloforms, the structures of which were deduced from discrete differences detected in molecular mass of the species separated by CE [10]. Using molecular masses calculated from the amino acid sequence and the type and amount of associated metals, it was possible to unequivocally identify distinct molecular forms.

The most definitive assessment of the metal composition of metalloproteins comes from the application of element-specific detection methods. CE–ICP–MS provides information not only about the type and quantity of individual metals bound to the proteins but also about the isotopes of each element as well [11,12]. Elemental speciation has become increasingly important to the areas of toxicology and environmental chemistry. Such analytical capability also opens up important possibilities for trace element metabolism studies. Figure 1 depicts the separation of rabbit liver metallothionein containing zinc, copper, and cadmium (the predominant metal) using CE–ICP–MS with a high-sensitivity, direct injection nebulizer (DIN) interface. UV detection (200 nm) was used to monitor the efficiency of the CE separation of the protein isoforms (MT-1 and MT-2), whereas ICP–MS detection made it possible to detect and quantify specific zinc, copper (not shown), and cadmium isotopes associated with the individual isoform peaks.

There are a number of emerging CE-based techniques that will greatly benefit the field of metalloprotein analysis in the near future. Major advances in in-

terfacing instrumentation that will result in more efficient separations and more sensitive detection in coupled systems, especially for CE–MS and CE–ICP–MS, are occurring now [9]. Further development of capillary electrochromatography (CEC), new column packing materials, and commercial systems that allow for gradient elution CEC will have a major impact on improving CE separations of metalloproteins. Moreover, coupling CEC to MS or ICP–MS detectors will offer new and more powerful ways to isolate and characterize metalloproteins. The ability to accurately detect and quantify elemental isotopes offers the promise of being able to conduct isotope dilution experiments involving human and animal subjects in which metal metabolism will be studied and the molecular (metalloprotein) level. Finally, the push toward miniaturization of CE instrumentation (CE on a chip) will find increasing application in the analysis of metalloproteins. This will be especially true in clinical/diagnostic laboratories, where sample size may be severely limited.

References

1. J. P. Landers (ed.), *Handbook of Capillary Electrophoresis*, 2nd ed., CRC Press, Boca Raton, FL, 1997.
2. T. Wehr, R. Rodriguez-Diaz, and M. Zhu, *Capillary Electrophoresis of Proteins* (J. Cazes, ed.), Chromatographic Science Series Vol. 80, Marcel Dekker, Inc., New York, 1999.
3. M. P. Richards and J. H. Beattie, *J. Capillary Electrophoresis* 1:196 (1994).
4. M. P. Richards and J. H. Beattie, *J. Chromatogr. B* 669:27 (1995).
5. M. P. Richards and T.-L. Huang, *J. Chromatogr. B* 690:43 (1997).
6. M. L. Shih and W. D. Korte, *Anal. Biochem.* 238:137 (1996).
7. T. Guszczynski and T. D. Copeland, *Anal. Biochem.* 260:212 (1998).
8. K. Haupt, F. Roy, and M. A. Vijayalakshmi, *Anal. Biochem.* 234:149 (1996).
9. K. L. Sutton and J. A. Caruso, *LC–GC* 17:36 (1999).
10. C. B. Knudsen, I. Bjornsdottir, O. Jons, and S. H. Hansen, *Anal. Biochem.* 265:167 (1998).
11. Q. Lu, S. M. Bird, and R. M. Barnes, *Anal. Chem.* 67:2949 (1995).
12. B. Michalke and P. Schramel, *J. Chromatogr. A* 750:51 (1996).