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Metalloprotein analysis by capillary isoelectric focusing[1]

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Abstract

Capillary isoelectric focusing (cIEF) was used to analyze three metalloproteins: conalbumin, transferrin and metallothionein (MT). Two different ampholyte mixtures were employed that generated linear pH gradients of 3–10 and 5–8. Several different proteins and one peptide with known isoelectric points (p*I*s) were used to establish linear relationships between peak migration time and p*I*. These standards were also used as internal markers to estimate peak p*I* values of the metalloproteins subjected to cIEF. Conalbumin (iron-free) subjected to cIEF with a pH gradient of 3–10 yielded a single major component (p*I* 7.17). When the protein was saturated with iron (2 Fe^{3+}/mol protein), a shift to lower p*I* was observed with a major peak (p*I* 6.24) and a lesser peak (p*I* 6.09). Mixing iron-free with iron-saturated conalbumin or adding iron to iron-free conalbumin prior to cIEF produced an additional peak (p*I* 6.68) that was presumed to be conalbumin containing a single iron atom (monoferric form). Human transferrin subjected to cIEF with a pH range of 3–10 gave a similar separation pattern to conalbumin with four major peaks at p*I* values of 6.25 (apotransferrin), 5.96 (monoferric form), 5.48 and 5.34 (diferric forms). Additional resolution of the molecular forms of both conalbumin and transferrin was achieved using a narrower pH gradient (5–8). Rabbit liver MT subjected to cIEF with a pH gradient of 3–10 gave a complex separation pattern with two prominent peaks (p*I* values of 3.73 and 3.56) that were presumed to be the fully metal-saturated MT-1 and MT-2 isoforms. When individual MT isoforms (MT-1 and MT-2) were separately subjected to cIEF with a pH gradient of 3–10, heterogeneous peaks with higher p*I* values (4.12–4.74) were observed. In contrast, horse kidney MT gave a single predominant peak with a p*I* of 4.09. MT samples could be separated using a pH gradient of 5–8 despite the fact that their apparent p*I* values were below the limits of the pH gradient established. In general, the heterogeneity observed for conalbumin, transferrin and MT proteins subjected to cIEF reflects the presence or absence of bound metal. Thus, cIEF represents a potentially useful analytical method which can provide information concerning the metal-binding characteristics of these and perhaps other metalloproteins.

Keywords: Metalloproteins; Conalbumin; Transferrin; Metallothionein

1. Introduction

Metalloproteins play many important roles in processes that are basic to cellular function such as respiration, metabolism and gene expression. Metals bound to these proteins serve either as catalytic or

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structural cofactors [1,2]. In order to fully characterize a particular metalloprotein, it is necessary to understand something about the binding of metal(s) to the protein. Different modes of capillary electrophoresis have been applied to the study of a variety of metalloproteins including: metalloenzymes such as carbonic anhydrase and alkaline phosphatase; heme-containing proteins such as hemoglobin and myoglobin; plasma metal-transporting proteins such as transferrin and albumin and intracellular metal storage proteins such as metallothionein (MT) and ferritin [3]. In particular, capillary isoelectric focusing (cIEF), applied to the separation of proteins, offers the advantages of speed and high resolution over more time-consuming isoelectric focusing (IEF) techniques that involve the use of columns or gels [4,5].

Previous work by Kilár and Hjertén [6–9] and Wu and Pawliszyn [10,11] has clearly demonstrated the feasibility of separating the molecular forms of transferrin that contain differing amounts of iron using cIEF. Transferrin, an iron-transporting protein present in blood plasma, is capable of binding up to two atoms of iron (Fe^{3+}) per molecule [12]. Thus, three distinct molecular forms are possible, those being: apotransferrin (no bound iron), monoferric transferrin (one bound Fe^{3+} atom), and diferric transferrin (two bound Fe^{3+} atoms). This has also been shown to be the case for conalbumin, the egg form of transferrin [13]. Moreover, since there are two iron-binding sites contained in the transferrin molecule, one N-terminal and one C-terminal domain, it was reported that IEF [12] and cIEF [5] could resolve two types of monoferric transferrin, depending on whether the iron was bound in the N- or C-terminal binding site. Thus, four distinct molecular forms of transferrin could be resolved by IEF based on the absence or presence of iron in the molecule. One objective of this study was to compare the separation of substantially iron-free, partially iron-saturated and fully iron-saturated forms of chicken egg white conalbumin and human serum transferrin by cIEF.

Metallothioneins (MTs) are a family of closely related, low molecular mass, heavy metal-binding proteins that are thought to play an important role in cellular metal metabolism [14]. Although MTs are generally recognized as acidic proteins [14], accurate estimation of their individual isoelectric points (p*I*s) has been complicated by the fact that the quantity and type of bound metal can influence the p*I*, by the fact that zinc and cadmium tend to dissociate from the protein in the pH range corresponding to the p*I* (3.9–4.6), and by the fact that MT proteins are prone to anomalous behavior during electrophoretic separation on polyacrylamide gels. Column-based IEF separation of MTs typically requires 24 h to complete and is problematic [15]. Gel-based IEF techniques also require lengthy separation times (17 h) and protein staining and gel scanning must be performed subsequent to the separation [16]. Current estimates of p*I* values for MT isoforms range from 3.9 to 10 depending on the source of the MT, its original metal composition and the conditions used for IEF [15]. Therefore, a second objective of this study was to attempt to estimate the p*I* values of two MTs, containing predominantly cadmium with lesser amounts of zinc derived from rabbit liver and horse kidney, using cIEF.

2. Experimental

2.1. Instrumentation

CIEF was performed on a P/ACE System 5510 (Beckman Instruments, Fullerton, CA, USA). A 27 cm length (20 cm to detector)×50 μm I.D. fused-silica neutral-coated capillary (eCAP Neutral Capillary; Beckman Instruments) was used. The capillary was housed in a cartridge with liquid cooling and the temperature was maintained at 20°C during the run. Data were collected and processed with System Gold software (Beckman Instruments).

2.2. Materials

Human serum transferrin (iron-poor, partially iron-saturated and iron-saturated); chicken egg white conalbumin (substantially iron-free and iron-complexed); Cd,Zn-MTs (rabbit liver and horse kidney) and the purified MT-1 and MT-2 isoforms (rabbit liver) were obtained from Sigma (St. Louis, MO, USA). Stock solutions of transferrin, conalbumin and MTs were prepared in deionized water at a concentration of 5.0 mg/ml. All cIEF reagents and

supplies including: polymer gel solution, ampholytes (pH range 3–10 and 5–8), 1.0 *M* phosphoric acid, 1.0 *M* NaOH, protein standards of known p*I* and the neutral-coated capillary were obtained from Beckman Instruments in kit form (eCAP cIEF 3–10 and eCAP cIEF 5–8 kits). Protein and peptide standards were dissolved in deionized water according to instructions supplied with each kit. The standards used were: ribonuclease A (p*I* 9.45), sperm whale myoglobin (p*I* 8.3), ACTH (p*I* 6.8), carbonic anhydrase II (p*I* 5.9), β-lactoglobulin A (p*I* 5.1) and CCK flanking peptide (p*I* 2.75).

2.3. Methods

cIEF was performed essentially as specified in the instructions provided with the kits. Prior to each run, the capillary was washed with deionized water for 1.0 min. The capillary was then filled with a mixture of ampholytes and proteins to be analyzed diluted into the polymer gel solution. For transferrin, conalbumin and MT analyses, appropriate standards were also included as internal p*I* markers. Proteins were focused by the application of an electric field strength of 500 V/cm (13.5 kV, for the 3–10 ampholyte mixture) or 700 V/cm (18.9 kV for the 5–8 ampholyte mixture) for 2.0 min. After 2.0 min the focused protein zones were mobilized past the detector by the simultaneous application of voltage (13.5 or 18.9 kV) and low pressure (3.5 kPa). The anolyte consisted of either 91 m*M* or 20 m*M* phosphoric acid in the polymer gel solution depending on whether the 3–10 or the 5–8 ampholyte mixture was used. The catholyte in all cases was 20 m*M* NaOH. Detection of transferrin and conalbumin was accomplished by monitoring UV absorbance at 280 nm, whereas, MTs were detected by monitoring UV absorbance at 254 nm. Estimates of p*I* were derived from linear regression analysis of the internal standard p*I* values versus their peak migration times. After each run the capillary was rinsed with 10 m*M* phosphoric acid followed by deionized water for 1.0 min each.

3. Results

Ribonuclease A (1), carbonic anhydrase II (2), β-lactoglobulin A (3) and CCK flanking peptide (4) with p*I* values ranging from 9.45 to 2.75 were well separated in less than 35 min using cIEF with a pH gradient of 3–10 (Fig. 1). An excellent linear relationship ($r^2 = -0.9962$) between p*I* and peak migration time (t) was determined using linear regression analysis ($pI = -0.31t + 12.92$). Similarly, a mixture of sperm whale myoglobin (A), ACTH (B), carbonic anhydrase II (C) and β-lactoglobulin A (D) were separated in about 30 min by cIEF with a narrower pH gradient of 5–8 (Fig. 2). Again, there was an excellent linear relationship ($r^2 = -0.9983$)

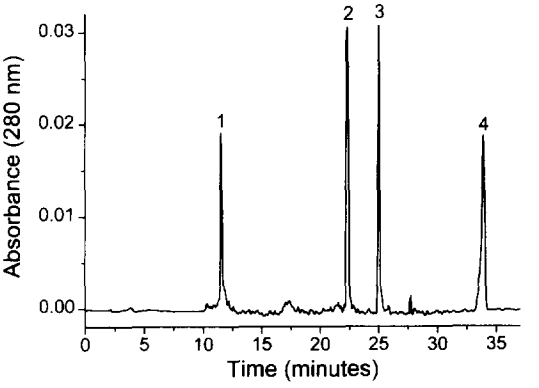

Fig. 1. Separation of four standard p*I* markers by cIEF with a pH gradient of 3–10. The standard markers were ribonuclease A (1), carbonic anhydrase II (2), β-lactoglobulin A (3) and CCK flanking peptide (4).

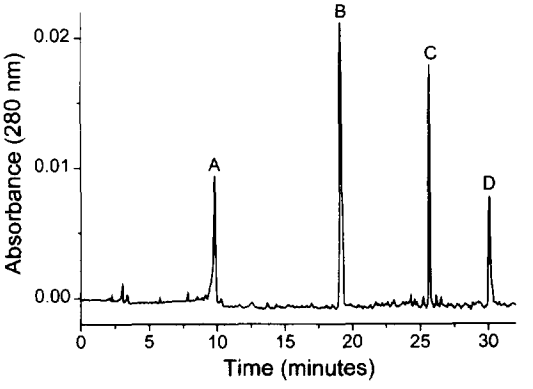

Fig. 2. Separation of four standard p*I* markers by cIEF with a pH gradient of 5–8. The standard markers were sperm whale myoglobin (A), ACTH (B), carbonic anhydrase II (C) and β-lactoglobulin A (D).

between p*I* and peak migration time (p*I* = −0.16*t* + 9.82). Thus, accurate estimates of p*I* values for sample proteins based on peak migration times can be obtained using internal standard protein and peptide markers with known p*I* values.

3.1. Conalbumin

Conalbumin (iron-free) separated by cIEF at pH 3–10 resulted in one major peak (p*I* 7.17) and several minor ones (Fig. 3A). Saturation of conalbumin with iron prior to cIEF produced two peaks with more acidic p*I* values (6.24 and 6.09) than observed for the iron-free protein peak, both of which were presumed to be diferric forms of the protein (Fig. 3B). The iron-free peak (p*I* 7.17) was absent from the iron-saturated conalbumin sample. When a mixture (2:1) of iron-free and iron-saturated conalbumin was subjected to cIEF at pH 3–10, an additional peak of intermediate p*I* (6.68) was detected (Fig. 3C) that was presumed to be conalbumin containing a single iron atom (monoferric conalbumin). This species may have been formed by iron-free conalbumin scavenging iron either directly from the diferric form (intermolecular transfer) or from residual iron, not bound to conalbumin, present in the iron-saturated preparation used in this study. We have also observed that the intermediate peak (p*I* 6.68) forms spontaneously when the iron-free conalbumin sample is stored in solution for extended periods of time (data not shown). Thus, it was necessary to prepare fresh samples of iron-free conalbumin just prior to analysis. These observations undoubtedly indicate the ability of conalbumin to acquire iron from its environment. In support of this concept, we found that the addition of an equimolar amount of iron (Fe^{3+} in dilute HNO_3) directly to

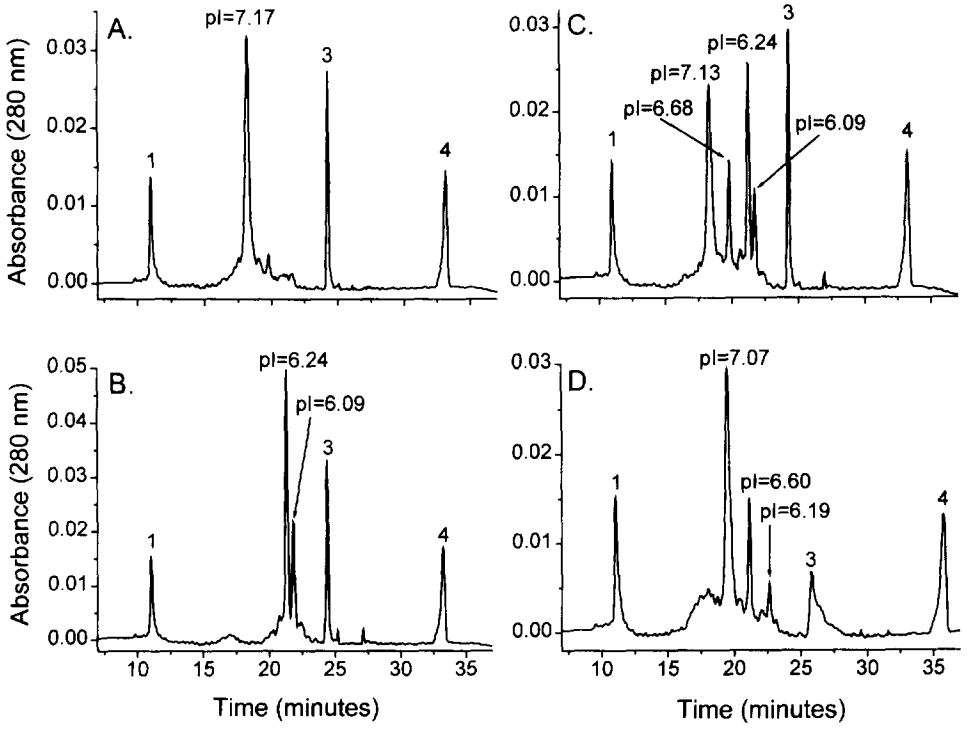

Fig. 3. Separation of substantially iron-free (A) and iron-saturated (B) chicken egg white conalbumin by cIEF with a pH gradient of 3–10. A mixture (2:1) of iron-free and iron-saturated conalbumin was also separated under the same conditions (C). An equimolar concentration of iron (Fe^{3+}) was added to iron-free conalbumin prior to cIEF and the mixture was then subjected to cIEF (D). The p*I* values of the conalbumin peaks are shown and were derived by linear regression analysis of data for three internal standard markers denoted by numbers (1, 3, 4) above the peaks corresponding to the markers listed in the legend to Fig. 1.

iron-free conalbumin prior to cIEF resulted in the formation of the monoferric as well as the diferric forms of the protein with the concomitant reduction in the iron-free peak (Fig. 3D). The presence of iron in the ampholyte/gel/sample mixture prior to cIEF also appeared to specifically affect the focusing of the β-lactoglobulin A internal standard (note shape of peak 3 in Fig. 3D). It also delayed the migration of the β-lactoglobulin A and the CCK flanking peptide internal standards (see peaks 3 and 4 in Fig. 3D) as well as the conalbumin peaks. This may account for the slight decrease in the estimated p*I* values for the conalbumin peaks compared to the previous separations in which iron was not added to the sample. Nevertheless, it is clear from the peak pattern that both monoferric and diferric forms of conalbumin were produced by the addition of iron to iron-free conalbumin (compare Fig. 3A with Fig. 3D).

3.2. Transferrin

Iron-poor (apo) transferrin, when subjected to cIEF with a pH 3–10 gradient, exhibited a single predominant peak (p*I* 6.25) with evidence of microheterogeneity (Fig. 4A). Iron-saturated transferrin separated by cIEF under the same conditions produced a number of peaks ranging in p*I* from 5.9–5.3 with a single predominant peak (p*I* 5.48) which is presumably a diferric molecular form of the protein (Fig. 4B). Transferrin partially (30%) saturated with iron separated by cIEF exhibited two predominant peaks with p*I* values of 6.24 (apotransferrin) and 5.48 (diferric transferrin) as well as a number of peaks with intermediate p*I* values (Fig. 4C). A peak (p*I* 5.96) that is presumably a monoferric molecular form of transferrin (possibly with iron bound to the C-terminal site) was present in the partially iron-saturated sample (Fig. 4C) and a lesser amount was

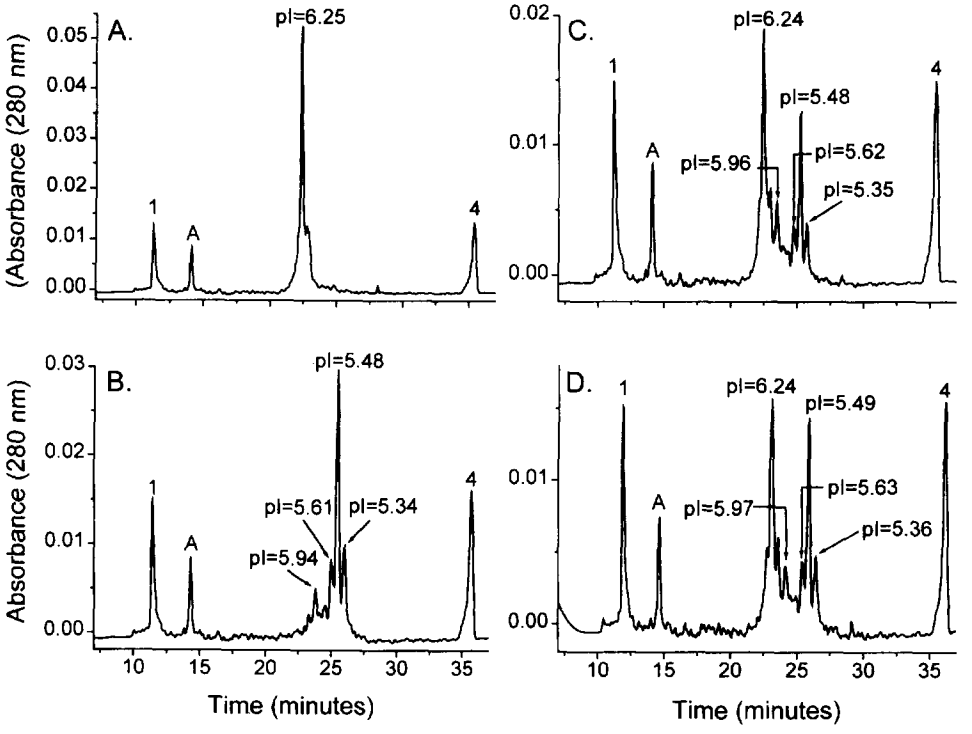

Fig. 4. Separation of iron-poor (A) and iron-saturated (B) human transferrin by cIEF with a pH gradient of 3–10. Partially (30%) iron-saturated transferrin was also separated under the same conditions (C). A mixture (1:1) of iron-poor and iron-saturated transferrin was prepared and subjected to cIEF (D). The p*I* values of the transferrin peaks are shown and were derived by linear regression analysis of data for three internal standard markers denoted by a number or letter (1, A, 4) above the peaks corresponding to the markers listed in the legends to Figs. 1 and 2.

also detected in the iron-saturated sample (Fig. 4B). Mixing equimolar amounts of iron-poor and iron-saturated transferrins produced a combined peak pattern indicative of the two samples (Fig. 4D). Unlike the mixture of conalbumins (Fig. 3C), there did not seem to be any increase in the monoferric peak (p*I* 5.97) as a result of combining the two transferrin samples.

In an attempt to achieve additional resolution of the molecular forms of both conalbumin and transferrin, a narrower pH gradient (5–8) was utilized for the cIEF separation (Fig. 5). It was not possible to adequately bracket conalbumin (Fig. 5A) or transferrin (Fig. 5B) with the available internal standard proteins to accurately estimate p*I* values under these conditions (pH 5–8). Therefore, internal standards were not included in the ampholyte/gel/sample mixture. It is clear from the separations depicted in Fig. 5A,B that with a pH gradient of 5–8 there was significant resolution enhancement of the molecular forms of both conalbumin and transferrin while still maintaining a pattern of peaks similar to that observed in the separations conducted with the 3–10 pH gradient (compare Fig. 3C and Fig. 4C with Fig. 5A and Fig. 5B). The separation of transferrin revealed another series of closely migrating peaks that could possibly constitute a second monoferric molecular form (with iron bound to the N-terminal site, $1Fe_N$) of the protein (Fig. 5B)

3.3. Metallothionein

Rabbit liver Cd,Zn-MT subjected to cIEF with a pH gradient of 3–10 gave a complex separation pattern containing two predominant peaks with p*I* values of 3.73 and 3.56 and a series of incompletely resolved peaks with p*I* values ranging from 4.0–5.0 (Fig. 6A). The two major peaks most likely are the fully cadmium-saturated (Cd_7) MT-1 (p*I* 3.73) and MT-2 (p*I* 3.56) isoforms. The unresolved series of peaks (p*I*s 4.0–5.0) may have resulted from the loss of metal (cadmium and/or zinc) from the MT proteins during focusing and mobilization thus giving rise to partially demetallated species which would be expected to exhibit higher p*I* values than fully metal-saturated MT-1 or MT-2 [17]. These peaks may also contain MTs with mixed (cadmium and zinc) metal content which would exhibit a higher p*I* value than MTs containing only cadmium [18,19]. For horse kidney Cd,Zn-MT, a different separation pattern was observed (Fig. 6B). A single major peak with a p*I* of 4.09 was observed. However, the shape of this peak strongly suggested the co-migration of the two major horse kidney MT isoforms (MT-1A

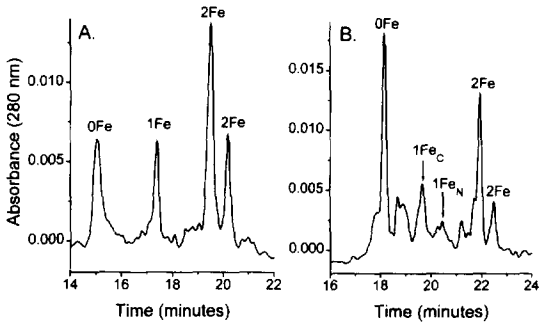

Fig. 5. Separation of a mixture (2:1) of iron-free and iron-saturated chicken egg white conalbumin (A) and partially (30%) iron-saturated human transferrin (B) by cIEF with a pH gradient of 5–8. The separated peaks are labeled as 0Fe, 1Fe and 2Fe to denote the apo (iron-free), monoferric and diferric molecular forms of these two proteins, respectively. For human transferrin (B), the monoferric molecular form was further subdivided and labeled as $1Fe_N$ and $1Fe_C$ to designate the presence of a single iron atom in the N- or C-terminal binding site, respectively.

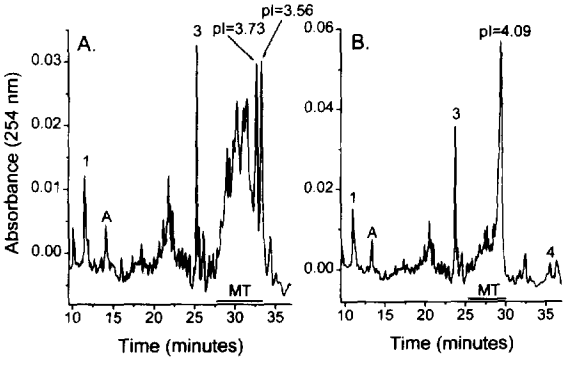

Fig. 6. Separation of rabbit liver Cd,Zn-MT (A) and horse kidney Cd,Zn-MT (B) by cIEF with a pH gradient of 3–10. The region containing MT peaks is denoted with the heavy line and the p*I* values of rabbit liver MT-1 (3.73) and MT-2 (3.56) isoforms and horse MT (4.09) are shown. Estimates of p*I* were derived by linear regression analysis of four internal standard markers denoted by a number or letter (1, A, 3, 4) above the peaks corresponding to markers listed in the legends to Figs. 1 and 2.

and MT-1B) both of which are known to carry two net negative charges [14]. Also observed were peaks with p*I* values ranging from 4.1–5.0 that were, again, most likely comprised of partially demetallated or mixed-metal MT species.

When individual MT isoforms from rabbit liver were subjected separately to cIEF, a different separation pattern was observed for both isoforms (Fig. 7A and Fig. 7B). The MT-1 isoform gave a predominant peak (p*I* 4.74) with a series of lesser abundant unresolved peaks (Fig. 7A). The MT-2 isoform exhibited two incompletely resolved peaks with p*I* values of 4.24 and 4.12 in addition to some lesser abundant and incompletely resolved peaks (Fig. 7B). In both cases, separation of the purified MT isoforms resulted in a series of peaks of higher p*I* than the two prominent peaks (p*I*s 3.73 and 3.56) observed in the intact MT sample (Fig. 6A). Although present in the MT-1 and MT-2 isoform samples, the two peaks of lower p*I* (3.73 and 3.56) were not major components in either of the two samples.

In an attempt to ascertain if the pH (especially pH≤4.0) affected the separation of rabbit liver and horse kidney MTs by promoting metal loss, cIEF was conducted using a pH gradient of 5–8 (Fig. 8). Despite the fact that the calculated p*I* values of both MT isoforms were below this range, both MT

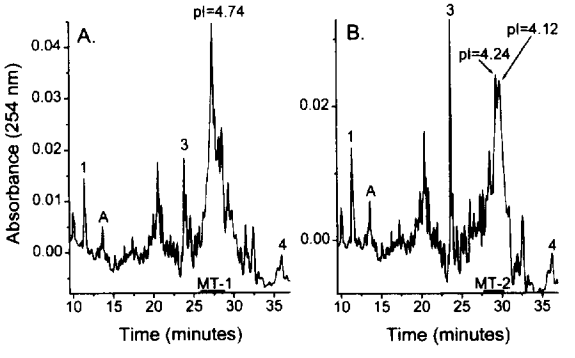

Fig. 7. Separation of purified rabbit liver MT-1 (A) and MT-2 (B) by cIEF with a pH gradient of 3–10. The MT isoform peaks are denoted with the heavy line and the p*I* values of the MT-1 (4.74) and MT-2 (4.24, 4.12) isoforms are shown. The p*I* estimates were derived by linear regression analysis of four internal standard markers denoted by a number or letter (1, A, 3, 4) above the peaks corresponding to markers listed in the legends to Figs. 1 and 2.

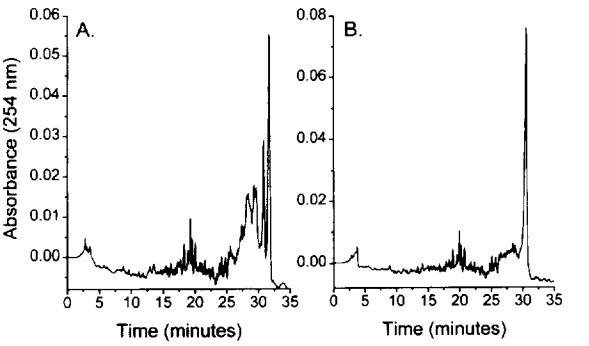

Fig. 8. Separation of rabbit liver Cd,Zn-MT (A) and horse kidney Cd,Zn-MT (B) by cIEF with a pH gradient of 5–8.

samples (rabbit liver and horse kidney) separated with a peak pattern similar to that observed when cIEF was conducted with a pH gradient of 3–10 (compare Fig. 6 with Fig. 8). In both cases, the peaks of lower p*I* appeared to be sharper and were now clearly predominant. Thus, one advantage of conducting the cIEF separation of MT isoforms with the pH 5–8 gradient appears to be reduced loss of metal from the proteins during the separation. To verify this possibility, a sample of rabbit liver MT was incubated with a 10-fold molar excess cadmium (Cd^{2+} in dilute HNO_3) prior to the cIEF separation with a pH gradient of 3–10. Under these conditions, the two peaks presumed to be the fully metal(cadmium)-saturated MT-1 and MT-2 isoforms became the predominant ones in the pattern of peaks resolved (Fig. 9). The separation pattern more closely resembled that of the rabbit liver MT separated with a pH gradient of 5–8 (Fig. 8A) than the separation conducted at pH 3–10 without added cadmium (Fig. 6A). This once again suggested that MTs containing a full complement of cadmium have lower p*I* values than the partially saturated or mixed metal proteins.

4. Discussion

The technique of cIEF offers the advantages of speed and high resolution over more time-consuming IEF techniques that utilize columns or gels [4,5]. Simultaneous application of low pressure and voltage has been previously reported to be an effective

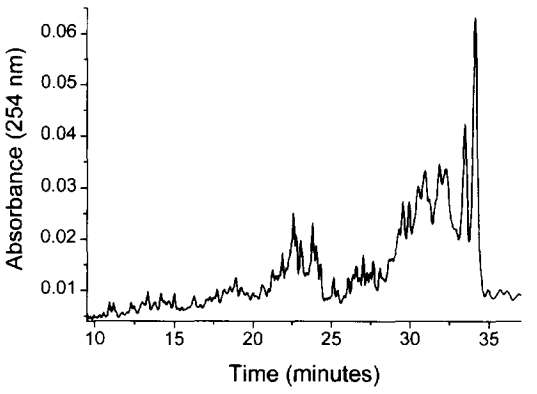

Fig. 9. Separation of rabbit liver Cd,Zn-MT to which a 10-fold molar excess of cadmium had been added prior to cIEF with a pH gradient of 3–10.

means for the hydrodynamic mobilization of focused protein zones in cIEF [20,21]. This study confirms those findings and clearly demonstrates an excellent linear relationship between migration time and p*I* for proteins separated using either a wide (3–10) or narrow (5–8) pH gradient. We have also shown that proteins and peptides of known p*I* can be effectively utilized as internal standards with cIEF to accurately estimate p*I* values of sample protein peaks as was reported previously [20]. In this study we applied cIEF to the analysis of three metalloproteins, two of which are glycoproteins (conalbumin and transferrin). Each of these proteins is known to exhibit heterogeneity based, in part, on the presence or absence of metal bound to the protein.

4.1. Conalbumin

This and a companion study [21] constitute the first applications of cIEF to the separation and characterization of conalbumin, a form of transferrin found in egg white. We report that three distinct molecular forms of conalbumin were present in varying amounts depending on the number of iron atoms bound. The estimated p*I* values of each of these were 7.17, 6.68 and 6.24 for the iron-free, monoferric and diferric forms, respectively. Our p*I* values are somewhat higher than those reported earlier for the iron-free (6.73), monoferric (6.25) and diferric (5.78) conalbumin separated using column-based IEF with a narrow pH gradient of 5–8 [13]. Interestingly, the incremental difference between the estimated p*I* values for the iron-free versus the monoferric form (0.49 versus 0.48) and the monoferric versus the diferric form (0.44 versus 0.47) are very similar for this study as compared to data reported previously [13]. This indicates that a similar degree of resolution of the molecular forms was achieved in both studies. The difference in absolute p*I* estimates may reflect differences in the IEF techniques employed which involved the use of internal p*I* markers (this study) as opposed to direct pH measurement on collected fractions [13]. The differences may also reflect the fact that the p*I* estimates were obtained using either a wide pH gradient (3–10, this study) or a narrow pH range (5–8) as reported previously [13]. Although conalbumin molecular forms were well resolved by cIEF using the 5–8 pH gradient, we were not able to accurately estimate p*I* values of the individual peaks separated under these conditions. This was due to a lack of suitable standards to employ as internal p*I* markers that would bracket, but not overlap, the peaks separated in this pH range. In general, there are only a limited number of appropriate internal protein or peptide standards for accurate p*I* estimation. This is a particular concern with the use of narrow pH gradients in cIEF. Perhaps, synthetic standard p*I* markers such as the substituted aminomethylphenol dyes could be used for this purpose [22,23].

The addition of iron to iron-free conalbumin resulted in a shift in the p*I* to a more acidic value which has been observed previously for both conalbumin [13] and transferrin [6,12]. We also observed a remarkable ability of conalbumin in solution to acquire iron from its surroundings which is consistent with its proposed function as a metal scavenger in egg white [24]. Kilár and Hjertén [6] reported that iron-free transferrin was able to acquire iron leached from the metal plunger of a microsyringe used to mix the protein with the ampholytes and this acquisition of iron led to the formation of monoferric and diferric transferrins during the mixing process. Similarly, when excess iron was added to iron-free conalbumin prior to cIEF, monoferric and diferric molecular forms (peaks with lower p*I* values) were subsequently detected (Fig. 3D).

The reason for the microheterogeneity observed in

iron-saturated conalbumin is not clear. However, Wenn and Williams [13] also reported two species (a predominant and a lesser abundant peak) characterizing iron-saturated conalbumin, both of which they classified as diferric forms. It is possible that this heterogeneity arises from variations in the carbohydrate chains attached to the conalbumin molecule [24] or in the amino acid sequence of the protein itself. Clearly, definitive identification of the peaks resolved by cIEF for conalbumin awaits further characterization either by alternative on-line detection techniques or by off-line techniques such as elemental analysis and mass spectrometry applied to fractions collected following cIEF.

Monitoring the absorbance at 460 nm has been described as an on-line detection method that is specific for the iron-bound molecular forms of transferrin separated by cIEF [6]. However, we were not able to successfully monitor absorbance at 460 nm to confirm the presence of iron in the putative monoferric and diferric conalbumin peaks separated in this study. Perhaps this was due to the narrow bore (50 μm I.D.) capillary used and the characteristics of our detector which did not give sufficient detection sensitivity at this wavelength. However, it still may be possible in future studies to employ this specific detection method (460 nm) simply by increasing the amount of protein applied to the capillary. This should produce focused zones of higher protein concentration assuming that the addition of more protein does not promote precipitation which can occur during the focusing step.

4.2. Transferrin

In this study we determined p*I* values for three molecular forms of human serum transferrin, those being: the iron-free (6.24), monoferric (5.96) and diferric (5.48) forms. Previously, p*I* values for the iron-free (6.1), monoferric (5.8–5.9) and diferric (5.5–5.6) forms were determined using gel-based IEF separations [12]. Similarly, using cIEF, p*I* values for the iron-free (6.10), monoferric (5.75–5.85) and diferric (5.45) molecular forms of human transferrin were also reported based on measured current values during the mobilization step [8]. Thus, there appears to be good agreement between the values determined in this study using a wide (3–10) pH gradient and those of other studies using narrower (5–7) pH gradients.

We found evidence for a single predominant peak (p*I* 5.96) that was presumed to be monoferric transferrin. In contrast, Kilár and Hjertén [6] have demonstrated that two monoferric molecular forms of transferrin could be separated using a narrow (5–7) pH gradient by cIEF. The two forms resulted from iron binding either to the N- or the C-terminal metal-binding site within the protein. Based on the findings of Kilár and Hjertén [6] and the p*I* value of the separated peak (5.96), we conclude that the predominant peak corresponding to monoferric transferrin detected in this study is the molecular form with iron complexed to the C-terminal binding site ($1Fe_C$, Fig. 5B). When separated by cIEF with a pH gradient of 5–8, the partially (30%) iron-saturated transferrin sample in this study did exhibit a second complex peak of intermediate p*I* between the iron-free and diferric peaks that could constitute a second monoferric form of transferrin corresponding to the form with a single iron atom bound to the N-terminal binding site ($1Fe_N$, Fig. 5B). It has been reported that the relative amount each of the two monoferric molecular forms of transferrin is determined by the method used to saturate the iron-free protein with iron [6]. Thus, our findings of a single predominant monoferric form of transferrin may simply reflect the specific method used to produce the partially iron-saturated protein.

The fact that transferrin showed considerably more microheterogeneity than conalbumin (see Fig. 5) most likely reflects the presence of sialic acid residues in the carbohydrate portion of the transferrin molecule. The number of sialic acid residues in transferrin can vary from 2 to 6, with the 4 sialoform being the most common [6,12]. Each sialic acid residue imparts an additional negative charge to the molecule and thus reduces its p*I* [6,12]. The iron-free, monoferric and diferric forms of transferrin would all be expected to show microheterogeneity due to differences in the sialic acid residue numbers. Thus, a mixture containing all of the molecular forms of transferrin would be expected to give a complex pattern of peaks when separated by cIEF. Since the carbohydrate attached to conalbumin reportedly contains no sialic acid residues [24], this could account for the less complex pattern observed

for the separation of this protein as compared to transferrin (see Fig. 5).

The degree of saturation of transferrin in blood is indicative of the iron status of an individual. Therefore, cIEF might represent a rapid and efficient method to directly assess the metal status of transferrin isolated from a blood sample. Assessment of transferrin iron-binding status based on the relative abundance of the different molecular forms of the protein could constitute an accurate and direct method to follow changes in iron status which would be useful for clinical diagnoses.

4.3. Metallothionein

This is the first report of the rapid and efficient separation of MT isoforms using cIEF. Previous IEF analyses of MTs separated from the tissues of mice, rats and humans involved time-consuming, column-based separations in which fractions were collected and the MT isoforms detected by analysis of bound metals or gel-based separations which involved staining of the focused protein bands with subsequent scanning of the gel [15–19]. In this study, detection was accomplished by direct monitoring of absorbance in the UV region (254 nm). Since MTs characteristically contain no aromatic amino acids [14], monitoring absorbance at 280 nm is not a viable method for detection. We relied instead on monitoring absorbance at 254 nm since it is known that the tetrahedral cadmiun–sulfhydryl complexes ($Cd–S_4$) of MT give rise to enhanced absorption at this wavelength due to the cadmium–sulfur charge transfer [25]. Despite the fact that monitoring UV absorbance below 280 nm resulted in a less stable baseline due to background absorbance by the ampholytes [4,5], it was still possible to easily detect cadmium-containing MT isoform peaks separated by cIEF.

MTs generally occur in tissues as zinc- and copper-containing proteins, with the majority of metal being zinc. It is currently not feasible to apply the cIEF technique described here to the separation and characterization of such proteins. It may, however, be possible to exchange the zinc with cadmium, since cadmium is known to have a higher affinity for MT than zinc [14]. In fact, cadmium saturation is a commonly performed method for both stabilization and quantification of MTs that can be applied to purified MT samples or MTs contained in more complex matrices such as tissue extracts [26]. Thus, it may be necessary to fully saturate MT samples to be separated with cadmium prior to cIEF. However, such a procedure would preclude any characterization of the native metal status of the MT samples subsequent to the cIEF separation.

We suspect, based on the separation of MTs with the pH 3–10 gradient, that metal may be lost during cIEF. Furthermore, we have shown that the addition of metal (cadmium) to the MT samples prior to cIEF or the use of a less acidic pH gradient (5–8) both affect the pattern of peaks separated. Since the ampholyte preparations typically used in IEF have the ability to chelate metals [5,6], these compounds could promote the exchange of zinc and/or cadmium with the MTs when first mixed with the protein sample or during the course of the separation in the capillary. The ampholytes used in IEF may also be a source of metal contamination for metalloproteins subjected to cIEF separations. Clearly, further work is required to establish whether or not metal loss or exchange is a problem for MTs separated by cIEF. We know that MTs subjected to free-solution capillary electrophoresis at neutral or alkaline pH retain their metals [27]. If it can be clearly established that the procedure of cIEF does not affect the native metal-binding status of MTs, then it may be possible to utilize this technique as a means to assess different metal-related heterogeneity in the population of MT molecules isolated from different tissues or different species of animals. This approach would require the use of MT standards of defined metal composition and appropriate methods for their detection.

In general, estimates of p*I* for MT isoforms are acidic and in the range of 3.9–6.0, with MT-1 having a higher p*I* than the more negatively charged MT-2 isoform [15]. The type of metal composition has been reported to influence the p*I* [18,19]. Our estimates of p*I* for rabbit liver MT-1 (3.73) and MT-2 (3.56) are lower than those previously reported for rabbit liver MT-1 (4.5) and MT-2 (3.9) isoforms [18]. However, when purified samples of MT-1 and MT-2 from rabbit liver were subjected to cIEF, higher estimates of p*I* (4.74 for MT-1 and 4.12–4.24 for MT-2) were obtained. It is not clear why the purified MT isoform preparations showed

only minor amounts of the peaks with lower p*I* (3.73 and 3.56) that were apparent in the intact rabbit liver MT sample. Perhaps, the procedure used to purify the individual isoforms (anion-exchange chromatography) modified them in some way (i.e., altered the metal composition or ratio). It is also possible that the isoforms are less stable in isolation than they are in combination. In addition, the purified samples may have contained residual salts that could interfere with focusing of the proteins during the cIEF separation. Recombining the two isoforms (MT-1 and MT-2) in equal proportions prior to cIEF did not generate additional amounts of the low p*I* peaks indicating that these peaks were characteristic of the intact sample (data not shown). Moreover, addition of cadmium to purified MT-1 and MT-2 isoforms prior to cIEF did not cause a shift in the peaks to lower p*I* as it did for the intact MT sample from rabbit liver (data not shown).

Horse kidney MT separated by cIEF yielded a single predominant peak (p*I* 4.09) which is consistent with the fact that both forms of equine MT (MT-1A and MT-1B) are known to share a common net negative charge that is characteristic of other mammalian MT-1 isoforms [14]. There was also a much smaller proportion of peaks detected migrating with higher p*I* values than was observed for the intact MT sample from rabbit liver. Perhaps, this reflects a greater stability of the structure of horse kidney MT as compared to rabbit liver MT. This might impart a greater resistance to low pH-induced metal loss or metal exchange that would cause a shift to the peaks with higher p*I* values.

The reason for the heterogeneity observed in MTs separated by cIEF, particularly MT from rabbit liver, is not clear. However, there are a number of possible factors that could account for the observed complexity in the separation pattern. Rabbit liver MT has been shown to be genetically polymorphic with seven isoforms identified and characterized, some of which differ in their net negative charge [28]. It is also possible that some of the complexity observed for MTs subjected to cIEF with a wide pH gradient (3–10) is due to loss of metal and subsequent polymerization either prior to or during the separation. Nordberg [15] has cautioned against exposure of MT samples separated by IEF to the acidic extreme portion of the gradient since this will cause loss of metal and thus affect p*I*. The fact that conducting cIEF with a narrower pH gradient (5–8) which did not expose the MTs to the lower pH extreme produced more prominent peaks at lower p*I* values indicates that metal loss could possibly explain some of the shift in peaks to higher p*I* during the run. Templeton and Cherian [17] found that the loss of one or two metal binding sites on rat liver MT-2 caused an increase in p*I* from 4.6 to 5.2. Conversely, it is well known that the binding of metals to apothionein (metal-free MT) results in an increased net negative charge and presumably decreased p*I* [14]. The MT samples separated in this study were mixed-metal MTs containing predominantly cadmium (4.8–7.3%, w/w) with lesser amounts of zinc (0.5–0.8%, w/w). Nordberg et al. [19] suggested that different p*I* values for MTs might be the result of different ratios of cadmium/zinc, with the purely cadmium-containing MTs (Cd_7-MT) exhibiting lower p*I* values than those of mixed metal composition (i.e., Cd_4Zn_3-MT). That is consistent with our findings for the addition of cadmium to rabbit liver MT causing a shift to peaks of lower p*I* (Fig. 9). The reason for this is not known. However, it is known that cadmium has a higher affinity for MT than zinc and will readily exchange with MT-bound zinc [14]. If it is assumed that cadmium-rich MTs are more stable than zinc-rich MTs or MTs of mixed metal composition, then stabilization of a particular molecular structure might account for these differences in p*I* values.

5. Conclusions

cIEF with combined pressure and voltage mobilization was used to rapidly and accurately characterize the p*I* values of different molecular forms of three metalloproteins: conalbumin, transferrin and metallothionein. It was possible to separate and detect three major molecular forms of conalbumin and transferrin presumably containing 0, 1 or 2 atoms of ferric iron. The iron-free form of conalbumin and transferrin exhibited higher p*I* values than the diferric form of both proteins. This information could be used to assess the degree of iron saturation of serum transferrin which would be indicative of iron status. Two isoforms from rabbit liver MT were

resolved and shown to have acidic p*I* values. The two comparable isoforms from horse kidney were found to co-migrate with a slightly higher, but still acidic p*I* values. Thus, by subjecting metalloproteins to cIEF, it is possible to derive useful information concerning metal-binding status of these molecules. It may even be possible to derive estimates of metal-binding affinities for different metalloproteins by adding varying amounts of a particular metal directly to the ampholyte/protein mixture prior to the separation of the different molecular forms by cIEF. In fact, Wu and Pawliszyn [11] have been able to observe the binding of iron included in the buffer during cIEF to apotransferrin and the subsequent formation of monoferric and diferric molecular forms using concentration gradient imaging detection. Affinity capillary electrophoresis (ACE) has proven to be a useful method for estimation of calcium association and dissociation constants for calmodulin [29] and this technique should be readily adaptable to metal-binding analyses of proteins separated by cIEF.

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