ELSEVIER

Journal of Chromatography A, 979 (2002) 249–254

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Evidence for oligomerization of metallothioneins in their functional state

Tore W. Wilhelmsen[a], Pål A. Olsvik[b], Bjørn Henrik Hansen[b], Rolf A. Andersen[b,*]

[a]*Pharmaceutical Department, Norwegian Medicines Agency, Sven Oftedalsvei 6, N-0950 Oslo, Norway*
[b]*Department of Zoology, Norwegian University of Science and Technology, N-7491 Trondheim, Norway*

Abstract

Capillary zone electrophoresis in the polyacrylamide-coated capillary was used to study metallothionein (MT) isoforms at physiological pH in horse kidney and rabbit liver MT preparations produced commercially by Sigma. Evidence is put forward that MT develops oligomers or aggregates in its metal binding situation at these pH values in both species. For the horse kidney preparation two forms were found for both the MT-IA and the MT-IB forms, for the rabbit liver three forms could be seen for the MT-I form and two for the MT-II form. At pH values above the physiological range (pH 8–10) up to four forms could be seen for the MT-I form (MT-IA in the horse) in both preparations. Compared to the MTCd-II form, the rabbit liver MTZn-II form not only behaved electrophoretically identical, but also showed a corresponding oligomerization behaviour. Our results indicate that the oligomerized MT-I form in the rabbit liver and the MT-IA form in the horse kidney bind more Cd atoms than the expected number of 7 per monomer.

Keywords: Metallothioneins; Proteins; Organosulfur compounds

1. Introduction

The metallothioneins (MTs) are small, cystein-rich metal-binding proteins, that participate in many protective responses towards stress in the organism. Although a single essential function of MT has not been demonstrated, higher eucaryotes may have evolved this protein to regulate levels and distribution of Zn in the organism. These proteins may also protect against heavy metals and oxidative stress inducing agents [1].

There are generally two major isoforms of MT in vertebrates, referred to as MT-I and MT-II. These proteins are similar with respect to sulfhydryl content and metal binding, but differ slightly in amino acid composition [2]. It is not known whether these similar, but obvious individual forms, play different roles in the organism. Previously interesting evidence has appeared that these MT isoforms may function as aggregates or oligomers in their functional in vivo state [3]. These aggregates may even bind a larger number of Cd atoms than suggested from the metal binding capacity of their individual monomer forms [4].

Such phenomena have been further studied for MTs from horse kidney in our laboratory [5]. In these studies we have used the capillary zone electrophoresis (CZE) technique, which has proved to offer an especially high separating ability of MT isoforms, including their oligomers. In the present

*Corresponding author. Fax: +47-7359-6311.
E-mail address: rolf.arvid.andersen@chembio.ntnu.no (R.A. Andersen).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)01259-1

work we have extended our studies to include corresponding phenomena occurring for the rabbit MT-I and -II forms at physiological pH values, also including the rabbit Zn containing MT-II form. These forms are commercially available. Our results indicate that oligomerization or aggregate formation of MTs is a general phenomenon.

2. Experimental

2.1. Chemicals

The MT preparations were obtained from Sigma, St. Louis, MO, USA: MT horse kidney (M4766), Cd 4.8%, Zn 0.8% (lot 73H9544); MT rabbit liver (M7641), Cd 6.7%, Zn 0.5% (lot 44H9568); MT-I rabbit liver (M5267), Cd 7.7%, Zn 0.9% (lot 24H9526); MT-II rabbit liver (M5392), Cd 5.7%, Zn 0.8% (lot 13H95481); MT-II Zn rabbit liver (M9542), Cd 0.1%, Zn 7.2% (lot 34H9548). These preparations were claimed to be essentially salt free. Sodium dodecyl-sulfate, Trizma–HCl and Trizma pre-set crystals (pH 9.1) were also bought from Sigma.

Orthophosphoric acid (H_3PO_4, 99% pure cryst.), HCl and EDTA (Titriplex III, ethylenedinitrilo-tetra-acetic acid dinatriumsalt-dihydrate, 99% purity) were bought from Merck (Darmstadt, Germany). Fluorinated liquid (FC-77) was obtained from 3M, Chemical Group (Haven, Belgium).

2.2. Capillary zone electrophoresis (CZE) of MT samples

The BioFocus 3000 capillary electrophoresis system with Spectra Software version 3.00, Integration Software version 3.01 and BioFocus capillary cartridge from Bio-Rad were used for MT analysis. Silica capillaries, HT-fused from MicroQuartz (Munich, Germany), 24 cm (19.3 cm to the detection window)×25 μm inner diameter were coated with polyacrylamide in our laboratory [6]. The coating solution was applied to the capillary by suction. To increase capillary durability they were kept at 4–6 °C. The MTs were dissolved in 10 m*M* Trizma pre-set crystal buffer at pH 9.1 to a final concentration of 180 μg/ml for the horse kidney MT, 250 μg/ml for the rabbit liver MT. These samples were kept as stock solutions at −80 °C under nitrogen.

The CZE experiments were performed in sodium phosphate (25–800 m*M*, pH 1.5–8.0) The individual experiments were done at constant voltage (8–20 kV) giving a current of maximum 60 μA in the capillary. This did not cause unwanted heat development in the capillary. All samples were applied hydrodynamically by pressure 20 (p.s.i.×s) and analysed with polarity from positive to the negative electrode below the isoelectric point (p*I*), the opposite way above p*I* (1 p.s.i.=6894.76 Pa). The cartridge and the carousel were thermostated at 20 °C by the Peltier thermoelectric cooling system with Fluorinated liquid. Prior to runs on the capillary column, the MT samples were kept in 10 m*M* Tris buffer at pH 9.1. In this way the change to running pH conditions occurred immediately before the actual run. At this high pH the metal will stay securely on the protein, thereby also protecting it from configurational changes and deterioration. The washing procedure for the system between each run was as follows: first 0.01 *M* HCl containing 0.5% sodium dodecylsulfate, then electrophoresis buffer for 30 s, and finally (from another tube) electrophoresis buffer for 60 s.

Absorbance was monitored at wavelengths of 200, 254 and 280 nm. Homogeneous Zn- and CdMT display optimal metal-thiolate absorption at 220 and 254 nm, respectively. The apoprotein generally absorbs below 240 nm and has a molar absorptivity less than for Zn–MT at 220 nm. In fact, 213 nm is reported to exhibit the greatest absorbance for the apoprotein. The metal therefore contribute significantly to the measured absorbances at the respective wavelengths. Absorption intensity at 254 nm was monitored to detect the mercaptide bond in MT, which causes absorption due to charge transfer between metal and sulfur bonding. Although this absorption can be detected in the case of the Cd–S bond, it cannot be observed in the case of the Zn–S bond [7–9].

3. Results and discussion

Figs. 1 and 2 show representative electropherog-

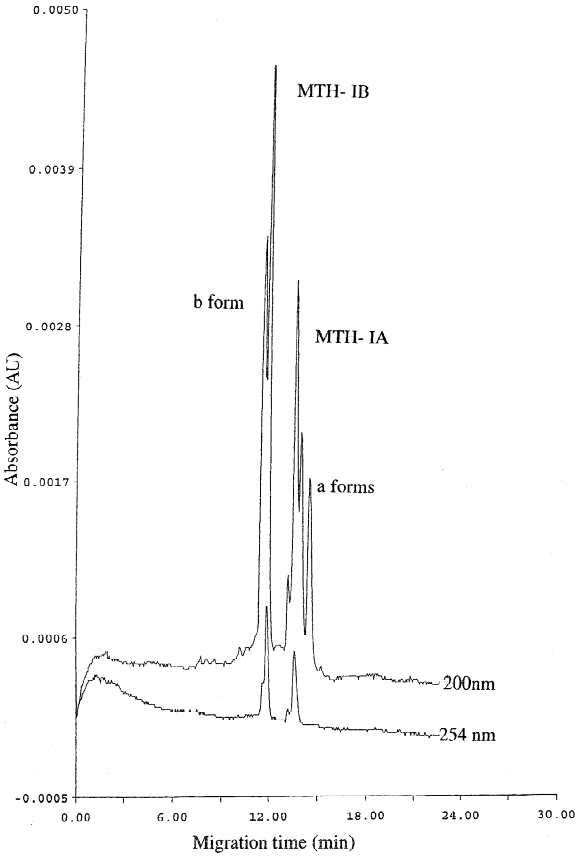

Fig. 1. A representative electropherogram for the horse kidney MT preparation run at physiological pH, showing two subforms of each main individual MT-IA and -IB isoform. In this case the small front IB subform is hidden by the b form. Running conditions: 15 kV, 60 μA, 50 m*M* phosphate buffer, pH 7.5. Electropherogram monitored at 254 nm represent Cd-thiolate binding.

rams run at pH 7.5 for the horse kidney and rabbit liver MT samples, respectively. These results were obtained for running conditions of 15 kV, 60 μA and 50 m*M* phosphate buffer, proving to be optimal for physiological pH in our polyacrylamide coated capillary. Runs performed at pH 7.0 showed almost identical electropherograms. A representative run at pH 1.5 of the rabbit liver MT preparation containing both the two isoforms MT-I and -II, is shown in Fig. 3. In this low pH range optimal running conditions were 10 kV, 55 μA and 500 m*M* phosphate buffer. For the horse kidney preparation the identification of the MT-IA,B peaks, as well as of the peaks representing the a and b forms was mainly based upon the absorbances monitored at 200 and 254 nm for which the presence of the cadmium mercaptide bond in MT could be detected only by the latter wavelength [5]. The rabbit liver MT-I and II peaks have been identified previously [10] and was further established in this work by running the electropherograms of each form separately as shown in Fig. 2b,c. The results show that the horse kidney MT-IB and the rabbit liver MT-II forms generally run faster in the capillary than the horse MT-IA and the rabbit MT-I forms, respectively. This is not unexpected due to the charge similarity between the horse kidney MT-IB and the rabbit liver MT-II forms [11–13].

The electropherogram obtained for the horse kidney MT-IA,B preparation at pH 7.5 is shown in Fig. 1. At this pH above the p*I*, metals are bound to the protein. Two main Cd binding MT forms are seen when monitored at 254 nm. A closer inspection of the two main peaks clearly shows that both were composed of two distinct metal binding subforms. A similar situation appeared for the mixed I and II isoform preparation from rabbit liver (Fig. 2a). The electropherogram of the rabbit MT-I form run individually, showed three subforms (Fig. 2b). For the individual MT-II form, however, only one form could be seen (Fig. 2c). Subform formation therefore seem to be a general trend occurring for MTs in both the horse kidney and rabbit liver. At pH values above the physiological range (pH 8–10) up to four forms could be distinguished both for the rabbit MT-I liver form and the MT-IA form from the horse kidney [5].

The main MT peaks identified in the present work at pH 7.5 both for the horse and rabbit MTs in the metal binding situation, are composed of oligomers or aggregates. This is shown previously in our lab in experiments involving addition of β-mercaptoethanol to the MT samples prior to capillary electrophoresis [5]. According to the expectation that this treatment should reveal monomeric forms, moving faster through the capillary, the MTs then appeared at the front of the electropherogram (not shown). Oligomerization or aggregate formation seem to be a general trend. This is also indicated by previous work [3,4,10,15–17]. The MT subforms observed may therefore represent oligomerization of mono-

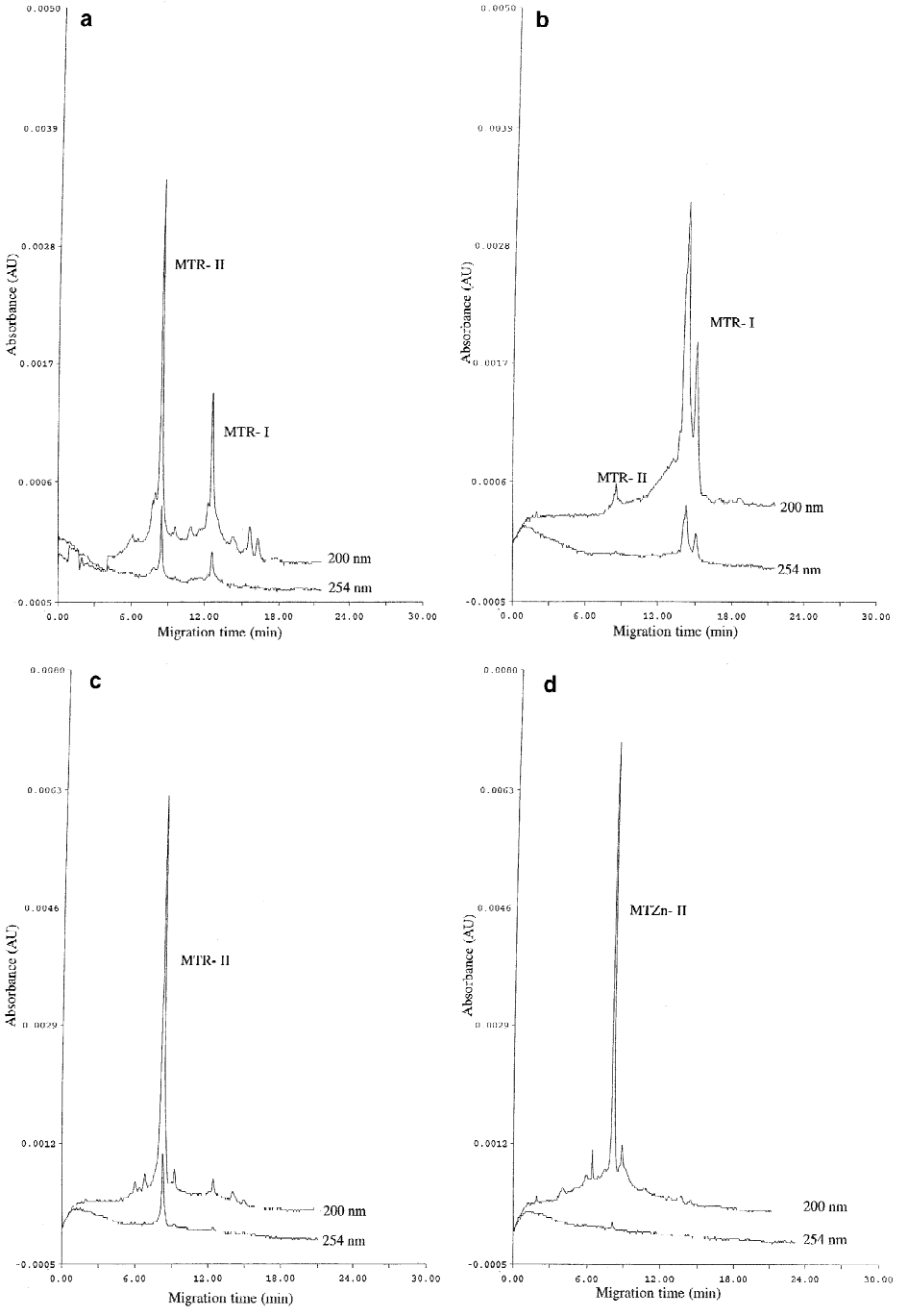

Fig. 2. Representative electropherograms for the rabbit liver preparations. Running conditions as in Fig. 1. (a) The mixed CdMT-I and -II isoform preparation, showing two subforms of each main individual form. A comparison between Figs. 1 and 2a shows that the main rabbit liver MT-II form behaves similar to the horse kidney MT-IB form in eluting ahead of the MT-I and the MT-IA form, respectively. (b) Electropherogram of the CdMT-I isoform, showing the presence of three MT subforms. (c) The CdMT-II isoform. In this case only one subform could be clearly distinguished. (d) The ZnMT-II isoform. Only one subform could be clearly identified, which is eluted similarly to the CdMT-II form. As expected the absorbance at 254 nm was nearly zero, because Zn-thiolates do not absorb at this wavelength.

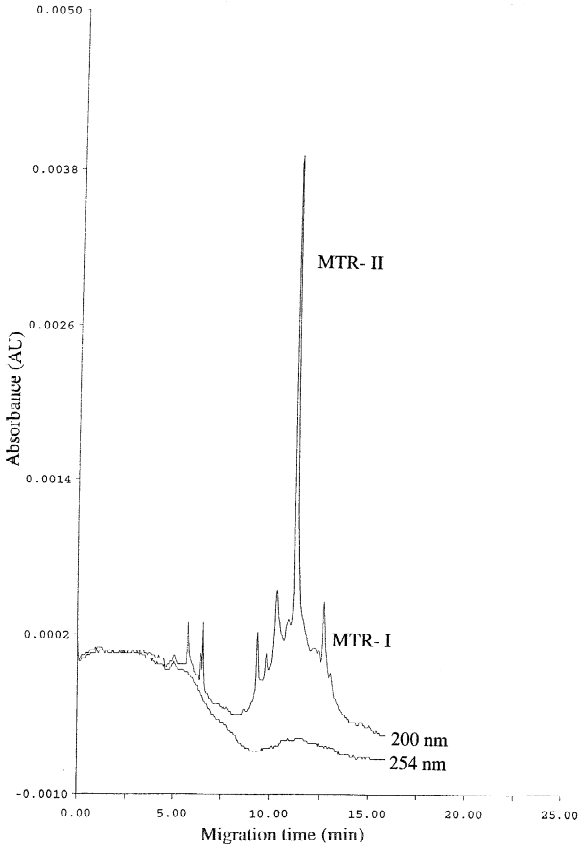

Fig. 3. The rabbit liver mixed MT-I and -II sample run at pH 1.5. This electropherogram shows a much smaller proportion of the MT-I form compared to the MT-II form than is shown from Fig. 2 where this sample was run at pH 7.5. See text for further discussion. Running conditions: 10 kV, 55 μA, 500 m*M* phosphate buffer. At pH 1.5, being well below the isoelectric point, the metals are stripped off the MT, thereby showing no absorbance at 254 nm.

mers in various proportions. It seems clear that such phenomena may be studied by the use of CZE.

The manufacturer (Sigma) informs that the rabbit liver ZnMT preparation is in fact the MT-II form induced by Cd injections into rabbits, from which the metal has been stripped off and subsequently replaced by Zn [14]. Replacement of Cd with Zn on the rabbit MT-II form obviously did not change the migration velocity in the capillary (Fig. 2c,d). Interestingly this replacement therefore did not seem to involve modifications of the MT, neither at the monomer nor at the oligomerized level.

Electropherograms of the horse kidney MT sample for pH values below the isoelectric point (p*I*) monitored at 200 nm in the metal deprived condition [5,10] indicated the presence of much more MT-IB than of the IA form. Above p*I*, however, the absorbance for the IA form at 254 nm, representing the Cd content [7–9], was much higher compared to the IB form. There may be various reasons for this, for instance that the IA form is considerably more unstable below p*I* and therefore disintegrates more easily in the metal deprived condition than the MT-IB form. Another possibility, however, is that the IA oligomers have an increased ability to bind additional Cd atoms than the expected number of 7 per monomer, thus giving a higher relative absorbance at 254 nm [4,5]. A similar relationship seem to exist for the rabbit liver MT-I form relative to the MT-II form. This is shown in the present work when Figs. 2a and 3 are compared. Since two new Cd atoms are included for each monomer added in the oligomerization [4], this would strongly increase the metal binding efficiency of the final protein aggregate, being a further argument that this is a general effect. The results in the present work indicate that the MT-I (horse IA) form is more involved in this process than the MT-II (horse IB) form.

Acknowledgements

The authors are grateful to Per Harald Olsen for valuable assistance with the graphics.

References

[1] C.D. Klaassen, J. Liu, S. Choudhuri, Annu. Rev. Pharmacol. Toxicol. 39 (1999) 267.
[2] J.H.R. Kagi, Methods Enzymol. 205 (1991) 613.
[3] K.T. Suzuki, M. Yamamura, Biochem. Pharmacol. 29 (1980) 689.
[4] P. Palumaa, E.A. Mackay, M. Vasak, Biochemistry 31 (1992) 2181.
[5] T.W. Wilhelmsen, P.A. Olsvik, R.A. Andersen, Talanta 57 (2002) 707.
[6] S. Hjerten, J. Chromatogr. 347 (1985) 191.
[7] M.J. Stillman, A.Y.C. Law, W. Cal, A.J. Zelazowsky, in: J.H.R. Kagi, Y. Kojima (Eds.), Metallothionein II, Experientia Suppl, Vol. 52, Birkhauser, Basel, 1987, p. 203.
[8] C.F. Shaw, M.M. Sawas, D.H. Petering, Methods Enzymol. 205 (1991) 401.

[9] K.T. Suzuki, Methods Enzymol. 205 (1991) 198.
[10] T.W. Wilhelmsen, P.A. Olsvik, S.W. Teigen, R.A. Andersen, Talanta 46 (1998) 291.
[11] J.H.R. Kagi, Y. Kojima, in: J.H.R. Kagi, Y. Kojima (Eds.), Metallothionein II, Experientia Suppl, Vol. 52, Birkhauser, Basel, 1987, p. 25.
[12] J.H.R. Kagi, S.R. Himmelhoch, P.D. Whanger, J.L. Bethune, B.L. Vallee, J. Biol. Chem. 249 (11) (1974) 3537.
[13] Y. Kojima, C. Berger, B.L. Vallee, J.H.R. Kagi, Proc. Natl. Acad. Sci. USA 73 (10) (1976) 3413.
[14] R.D. Comeau, K.W. McDonald, G.L. Tolman, M. Vasak, F.A. Liberatore, Prep. Biochem. 22 (1992) 151.
[15] D.T. Minkel, K. Poulsen, S. Wielgus, C.F. Shaw, D.H. Petering, Biochem J. 191 (1980) 475.
[16] R.A. Andersen, H.L. Daae, Comp. Biochem. Physiol. 90B (1988) 59.
[17] J. Hidalgo, J. Bernues, D.G. Thomas, J.S. Garvey, Comp. Biochem. Physiol. 89C (1988) 191.