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Effects of redox conditions and zinc(II) ions on metallothionein aggregation revealed by chip capillary electrophoresis

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ABSTRACT

Metallothioneins (MTs) belong to cysteine-rich proteins with unique higher structure. One of the most known MT's functions is metals detoxification and maintaining their homeostasis in a cell. Structure of MT with naturally occurred zinc(II) ions can be affected by concentration of metal ions as well as redox milieu inside a cell, however the exact explanation and biochemical effects of the structural changes are still missing. In this study we used capillary electrophoresis on chip coupled with fluorescence detection to determine structural changes of MT with increasing concentration of zinc(II) ions and under various redox conditions. To investigate the structural-dependent effects, reduced and/or oxidized apo-MT (MT without natural occurred metal ion) was prepared. Zinc binding into reduced and/or oxidized apo-MT was compared. MT was incubated with 0, 5, 15, 25, 50 and 100 μM $ZnCl_2$ for 1 h in 37 °C. Formation of MT aggregates with increasing zinc concentration was observed by spectrophotometry, chip capillary electrophoresis, and SDS-PAGE. We found out that reduced MT forms aggregates more readily compared to oxidized MT. Using the chip capillary electrophoresis allowed us relative quantification of MT aggregation as a decrease in the area of the signal corresponding to the monomer form of MT (Mw 15 kDa, migration time 26.5 s) and its ratio to total signal (sum of all signals measured by the electrophoresis). The dependences had an exponential character with equation $y = 2.4 \times e^{-0.01x}$, $R^2 = 0.945$ for 15 kDa peak area and $y = 0.11 \times e^{-0.01x}$, $R^2 = 0.938$ for decrease of 15 kDa peak area ratio to the total signal. Zn–MT interaction was 30% faster during the first 15 min and 50% faster during the whole experiment for reduced MT. It can be concluded that formation of MT aggregates is dependent on redox state and Zn(II) concentration.

1. Introduction

In 1957, rich in cysteine protein able to bind cadmium was isolated from horse kidney and named as metallothionein according to its structural properties [1]. Further, this protein and metallothionein-like proteins were found in tissues of other animal species, plants, fungi and even in yeasts [2–4]. The highest metallothionein (MT) content has been found in the tissues with the highest level of heavy metal, especially liver, kidney and spleen [5]. It was also shown that expression of MT is inducible by heavy metals [2,3,6,7].

The relative molecular mass of MT ranges from 2000 to 13,000 depending on a specie. The number and position of cysteine residues in metallothioneins structure are highly conservative and typical structural motifs cys-X-cys, cys-cys and cys-X-X-cys are formed (X and Y are aminoacid residues different from cysteine). Due to these motifs the binding α and β domains are able to chelate heavy metal ions. The domains differ in reactivity and heavy metal content. One MT molecule is able to bind up to 7 divalent and up to 12 monovalent metal ions. The affinity of MT to relatively abundant heavy metal ions decreases in order Hg(II) > Ag(I), ~Cu(II) > Cd(II) > Zn(II). Although MT exhibits the highest affinity for Cu(I), under physiological conditions most frequently Zn(II) ions are bound. Toxic heavy metal ions (e.g. Cd(II), Pb(II), Hg(II)) are capable to displace Zn(II) from MT structure due to their higher affinity to –SH moieties.

The first discovered and still not fully understood roles of MT are transporting of essential heavy metals and detoxification of the toxic ones. Thanks to thiol moieties from cysteine residues MT has markedly antioxidant properties. In synergy with glutathione (GSH) it is able to scavenge reactive oxygen species (ROS). Interestingly, MT level and stage and/or grade of malignant tumours are given into the relationship. Moreover, MT is likely to be involved in resistance against platinum-based cytostatics. These issues have been several times reviewed [8–10].

MT function is closely connected to its structure and oxidative state. A connection between MT redox state and aggregates formation as a response to heavy metals and redox conditions in

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organelles has been found. MT containing heavy metals, especially zinc, form covalent aggregates, which can be involved in subcellular retention and/or storage of the protein, but the physiological importance of the aggregation remains still unclear [11]. Based on the results published it can be concluded that MT can exist in forms varying in the affinity to zinc(II) ions, oxidation state and degree of polymerization. Polymerization can be a way of retaining MT in subcellular compartment, protecting it from degradation, or storing it to provide chelating or reducing capacity without a need for *de novo* synthesis of the protein [11]. Naturally occurring MT polymerization was observed also in rats, dogfishes, horses and rabbits [12,13].

Demuynck et al. used gel permeation chromatography for MT determination in earthworms exposed to Cd. They obtained fractions with molecular masses from 45 to 60 kDa, in which proteins with immunoreactivity to MT antibody were found. These molecular masses refer to MT aggregates [17,18]. Heavy-metal induced aggregation is well known also for other proteins. This process can be of great importance in a cell. Tougu et al. found that Zn(II)- and Cu(II)-induced non-fibrillar aggregates of amyloid-beta (1–42) peptide are transformed to amyloid fibrils, both spontaneously and under the influence of metal chelators [14]. On the other hand plant peptides called phytochelatins (PC) can aggregate to detoxify heavy metals. Lavoie et al. found that freshwater green algae, *Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata*, exhibited the different sensitivity to Cd. Subcellular Cd distribution and the synthesis of metal-binding thiolated peptides were thus examined in both algae exposed to Cd(II). Cadmium accumulation per cell was higher for *C. reinhardtii* than for *P. subcapitata*. Except an increase in the content of Cd, both species produced higher levels of PC, however, longer oligomers were found in *C. reinhardtii* [15].

To study MT aggregates accurate and robust methods are needed. Capillary electrophoresis (CE) is frequently used for separation of MT isoforms. Beattie firstly used CE for this purpose [16]. Using of CE for detection of MT has been summarized in several reviews [17–22]. Analysis of MT isoforms [16,23–25], modifications [26], monitoring of MTs expression [18,27,28], stability [29,30], studying of MT oligomerization [13,31] and heavy-metal binding into MT molecules [30,32–38] belong to the mostly investigated. This technique is also suitable for MT quantification in biological sample [18,24,31,32,39–42]. In spite of the great research of MT, the importance of structural changes, mainly forming of aggregates, is still not clear. The current accepted opinion is that MT aggregates somewhat associate with the presence of reducing and/or oxidizing agents and could be, thus, connected with pathophysiological processes in the cell. Therefore the aim of this study was to determine structural changes of MT under various experimental conditions including increasing concentration of zinc(II) ions and various redox conditions. All changes were studied by chip capillary electrophoresis and confirmed with spectrometry.

2. Experimental

2.1. Chemicals

All chemicals of ACS purity used were purchased from Sigma Aldrich Chemical Corp. (Sigma–Aldrich, USA), unless noted otherwise. Deionised water underwent demineralization by reverse osmosis using the instrument Aqua Osmotic 02 (Aqua Osmotic, Tisnov, Czech Republic) followed by further purification using Millipore RG (Millipore Corp., USA, 18 MΩ) – MilliQ water. The pH was measured using WTW inoLab pH meter (Weilheim, Germany).

2.2. Metallothionein reduction and heavy metals removal

In order to obtain quantitatively reduced apo-MT (MT without natural occurred metal ion), MT (2 mg/ml, Sigma–Aldrich, Cat. No. *M 7641*) was incubated in buffer containing 1 mM tris(2-carboxyethyl)phosphine and 1 mM EDTA, pH 3.0 (E/T buffer) for 1 h under shaking (1000 rpm) at 37 °C. Redox state and metal content were determined spectrophotometrically within the range from 200 to 300 nm, as a blank E/T buffer was used. Measurements were carried out with by UV–vis spectrophotometer Specord 210 (Analytik Jena, Germany). Experimental conditions were adopted according to papers [43,44] and further optimized. After the incubation the low-molecular mass molecules were removed by buffer exchange using Amicon® centrifugal filter device (recovery higher than 95% for 12.5 kDa protein and less than 42% for vitamin B-12 (Mr – 1350)) (Millipore Corp., USA). The obtained apo-MT was used for all following experiments.

2.3. Metallothionein interaction with $ZnCl_2$

MT interaction with Zn(II) was monitored spectrophotometrically by thermostated UV–vis spectrophotometer Specord 210 (Analytik Jena, Germany) at 37 °C (Julabo, Germany). The absorption spectra within the range from 200 to 300 nm were measured in 2 min intervals. *Interaction of reduced MT with Zn(II).* Reduced MT (0.2 mg/ml in 0.2 M phosphate buffer pH = 7.4) was incubated with 0, 5, 15, 25, 50 and 100 mM $ZnCl_2$ for 1 h at 37 °C. *Interaction of oxidized MT with Zn(II).* MT was oxidized by 0.5 or 1 μM $K_2Cr_2O_7$, and 0.5 or 1 μM $KMnO_4$ for 1 h under shaking (1000 rpm) at 37 °C. The oxidized MT was treated with $ZnCl_2$ (66 mM). Data were collected and analyzed by software package WINASPECT (Analytik Jena, Germany).

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The electrophoresis was performed according to Laemmli [45] using a Mini Protean Tetra apparatus with gel dimension of 8.3 cm × 7.3 cm (Bio-Rad, USA). First 15% (m/V) running, then 5% (m/V) stacking gel was poured. The gels were prepared from 30% (m/V) acrylamide stock solution with 1% (m/V) bisacrylamide. The polymerization of the running or stacking gels was carried out at room temperature for 45 or 30 min, respectively. To achieve repeatable results the gels were kept in the dark at 4 °C overnight. Prior to analysis the samples were mixed with reduction (7.5% β-mercaptoethanol) or non-reduction sample buffer in a 2:1 ratio. The samples were boiled for 2 min, and then 800 ng of MT was loaded onto a gel. For determination of the molecular mass, the protein ladder "Precision plus protein standards" from Bio-Rad was used. The electrophoresis was run at 150 V for 1 h (Power Basic, Bio-Rad, USA) in tris–glycine buffer (0.025 M Trizma-base, 0.19 M glycine and 0.0035 M SDS, pH = 8.3). Silver staining of the gels was performed according to Oakley et al. [46].

2.5. Capillary chip electrophoresis

Analyses on an automated microfluidic Experion electrophoresis system (Bio-Rad, USA) were carried out according to the manufacturer's instructions with supplied chemicals (Experion Pro260 analysis kit, Bio-Rad) with the following modifications: the sample buffer was diluted in ration 1:1 with water and the concentration of fluorescence dye in gel was lowered to one half. Those modification dramatically improved capability of the system to detect low amounts of MT. Except preparation of calibration curve, 800 ng of MT was used for all analyses. A sample (4 μl) was mixed with 2 μl of non-reducing sample buffer, and after 4 min boiling, 84 μl of water was added. After the priming of the chip with the

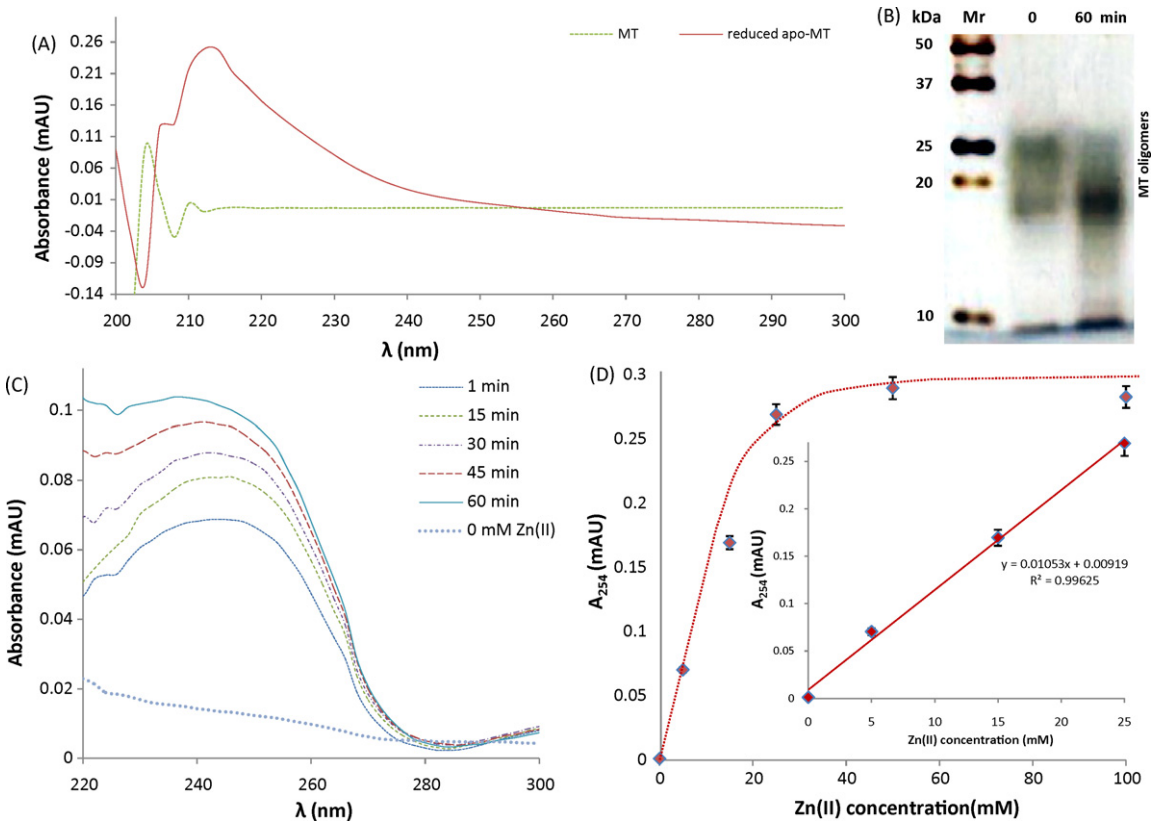

Fig. 1. (A) Comparison of the absorption spectra of metallothionein (0.2 mg/ml, 0.03 mM) within the range from 200 to 300 nm before (MT) and after reduction and metals removal (reduced apo-MT). (B) SDS-PAGE of 800 ng MT and reduced apo-MT. (C) Absorption spectra of 0.03 mM reduced apo-MT with 5 mM Zn(II) after 0, 1, 15, 30, 45 and 60 min interaction. (D) Interaction of 0.03 mM reduced apo-MT with zinc, dependence of the absorbance at 254 nm (A_{254}) on Zn(II) concentration after 15 min interaction, in inset: linear part of the dependence of A_{254} on Zn(II) concentration after 15 min interaction.

gel and gel-staining solution in the diluted priming station sample, the mixture (6 μl) was loaded into the sample wells. The Pro260 Ladder included in the kit was used as a standard.

2.6. Descriptive statistics

Data were processed using MICROSOFT EXCEL® (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean ± standard deviation (SD) unless noted otherwise (EXCEL®). Statistical significances of the differences were determined using STATISTICA.CZ. Differences with $p < 0.1$ were considered significant and were determined using *T*-test, which was applied for means comparison.

3. Results and discussion

3.1. Capillary chip electrophoresis

Capillary chip electrophoresis was firstly utilized for analysis of MT by Krizkova et al. [47]. The authors showed that the instrument could be used to study influence of oxidation on structure of MT, however, optimization of the sensitivity and detection limit as well as more precise investigation of the effect of oxidation agents on MT aggregation of the method was missing. Therefore, we attempted to better characterize the process of aggregation and also to optimize detection of MT. To more precisely and sensitively quantify MT by chip capillary electrophoresis the manufacturer's instructions was modified (details are shown in Section 2). Lowering of the concentration of the fluorescent dye and amount of sample buffer allowed us to lower MT detection limit for more than thirty folds compared to previous results, where chip capillary electrophoresis was used to study MT oxidation with H_2O_2 [47]. The signal height corresponded to MT was counted as a sum of total peaks areas within the range from 10 to 100 kDa. Under the modified conditions we obtained a linear calibration curve within the range from 125 to 1 000 ng MT with equation $y = 0.015x + 14.04$, $R^2 = 0.98$ (relative standard deviation below 5%, $n = 3$). Detection limit of MT was estimated as 20 ng.

3.2. Investigation of zinc(II) binding to reduced apo-metallothionein

To study Zn(II) binding into MT structure, reduced apo-MT was prepared. The yield of the reduction and zinc removal was monitored by measuring absorbance within the range from 200 to 300 nm. We observed an increase in peak with maximum at 212 nm, which corresponds to apo-MT [11,44]. The apoprotein generally absorbs below 240 nm and has a molar absorptivity less than for MT–Zn at 220 nm. Similar results were obtained by Wilhelmsen et al. [31]. The spectra of MT and reduced apo-MT are shown in Fig. 1A. The changes were also well detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Fig. 1B).

Prior to the following experiments E/T buffer was removed by ultrafiltration in the presence of 0.2 M phosphate buffer (pH 7.4). It is well known that apo-MT regain metal binding ability when pH exceeds above isoelectric point (p*I* = 3.9 for MT-1 and 4.5 for MT-2) [31]. Further reduced apo-MT was incubated in the presence of zinc(II) ions (0, 5, 15, 25, 50 and 100 mM) for 1 h at 37 °C. The binding of them into MTs structure was monitored spectrophotometrically. We observed an increase of the absorbance within the range from 212 to 284 nm with maximum at 254 nm, which is typical for heavy-metal binding into the MT structure [31,48]. Lower absorbance

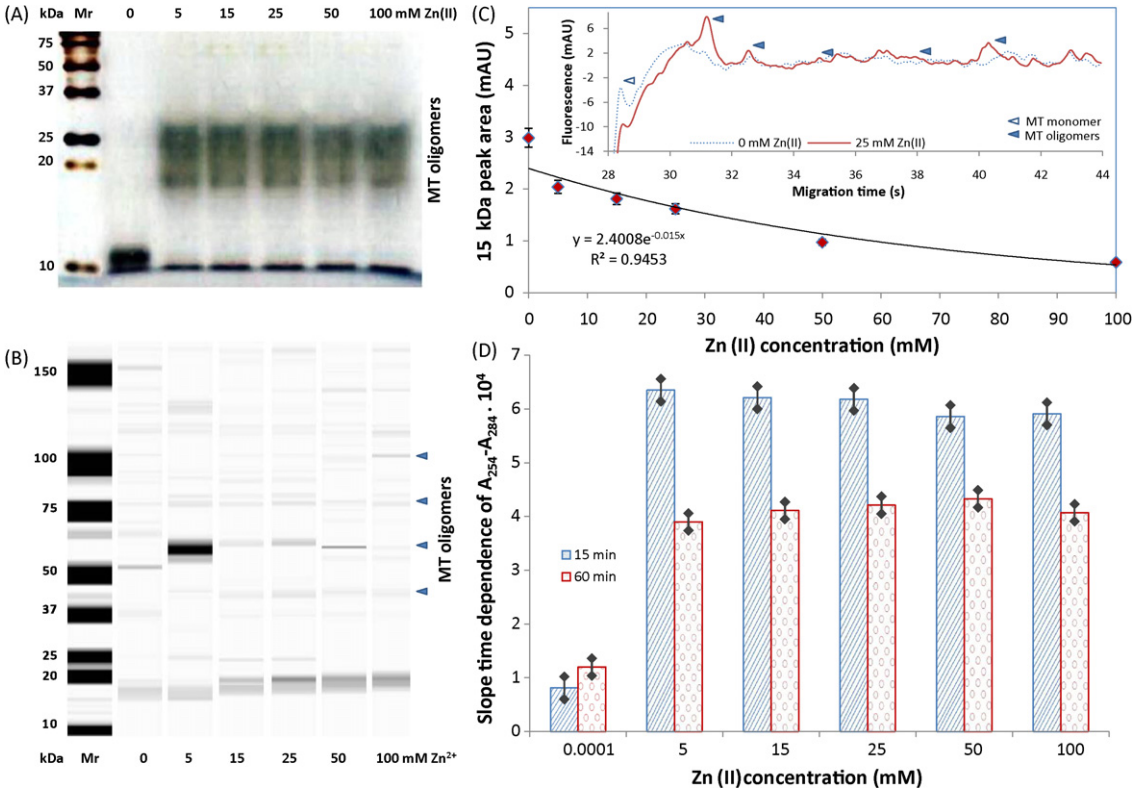

Fig. 2. Interaction of 0.03 mM reduced apo-MT with zinc. (A) SDS-PAGE of 800 ng reduced apo-MT after 60 min interaction with 0, 5, 15, 25, 50, and 100 mM Zn(II). (B) Virtual gel output from Experion system, 800 ng reduced apo-MT after 60 min interaction with 0, 15, 25, 50, and 100 mM Zn(II), arrows indicate MT oligomers. (C) Dependence of 15 kDa peak area on Zn(II) concentration, in inset: capillary electrophoreograms of 0.03 mM reduced apo-MT after 60 min interaction with 0 and 25 mM Zn(II), white arrow indicates MT monomer and full arrows indicate MT oligomers. (D) Slopes of $A_{254} - A_{284}$ time dependence (dA/dt) with increasing Zn(II) concentration within the range from 0 to 15 min (hatched bar) and from 15 to 60 min (dotted bar).

measured at 280 nm can be attributed to the fact that MT usually do not contain aromatic aminoacids [31]. The spectra of 5 mM Zn(II) depending on interaction time are shown in Fig. 1C. To evaluate zinc binding into MT structure the absorbance at 284 nm (A_{284}) was subtracted from the absorbance at 254 nm (A_{254}). The obtained net absorbance ($A_{254} - A_{284}$) was used for construction of time and concentration dependence of Zn(II)–MT interaction. Within the range from 0 to 25 mM Zn(II) we observed a linear dependence with equation $y = 0.01053x + 0.00919$, $R^2 = 0.996$, the dependences were same during the 60 min experiment. At higher concentration no further increase in absorbance was observed probably because of MT saturation (Fig. 1D).

MT aggregates of molecular mass from 20 to 30 kDa are shown in Fig. 2A. Similar results were obtained on chip capillary electrophoresis, where MT in di-, tri-, tetra-, penta- and hexameric form was observed (migration times 30.5, 33, 34.8, 38 and 41.5 s, Mw 24.6, 37, 47, 60 and 78 kDa). The virtual gel output is shown in Fig. 2B. Moreover, using the chip capillary electrophoresis allowed relative quantification of MT aggregation as a decrease in the area of the signal corresponding to the monomer form of MT (Mw 15 kDa, migration time 26.5 s) and its ratio to total signal (sum of all signals measured by the electrophoresis). The dependences had an exponential character with equation $y = 2.4 \times e^{-0.01x}$, $R^2 = 0.945$ for 15 kDa peak area and $y = 0.11 \times e^{-0.01x}$, $R^2 = 0.938$ for decrease of 15 kDa peak area ratio to the total signal (Fig. 2C). After the exclusion of 0 mM Zn(II) concentration the dependences had shapes $y = 2.16 \times e^{-0.01x}$, $R^2 = 0.98$ for change in signal of monomeric MT form $y = 0.102 \times e^{-0.01x}$, $R^2 = 0.964$ for change in ratio of monomeric MT form to total signal. In addition, formation of MT aggregates was determined as a change in $A_{254} - A_{284}$ value. We were able to split the signal increase dependence on time into two linear section (0–15 and 16–60 min) with reliability coefficients higher than 0.96. The slopes exhibit the rate of zinc binding into the MT structure. For the first part of the dependence (0–15 min) the average value was $6.08 \times 10^{-4} \pm 2.31 \times 10^{-5}$, and for the second part (16–60 min) the average value was $4.13 \times 10^{-4} \pm 1.85 \times 10^{-5}$. Whether we measured reduced apo-MT without Zn(II), the values of the slopes were 8.1×10^{-5} and 1.2×10^{-4} (Fig. 2D). Differences between the slopes were with $p < 0.1$ and thus were considered significant.

It can be concluded that Zn–MT interaction is 30% faster during the first 15 min. This phenomenon suggests the MT saturation at higher durations of the interaction. The increase of the $A_{254} - A_{284}$ value suggests formation of new Zn-binding sites most likely due to MT aggregation. Increased ability of metals binding into MT in aggregated form was previously shown by several authors [31,48,49], which supports our hypothesis.

3.3. Investigation of zinc(II) binding to oxidized apo-metallothionein

MT redox state is essential for its function as a scavenger of ROS. One of the effects of toxic heavy metals is induction of free radicals damaging cell compartments. Therefore we were interested in the issue how the zinc binding ability of reduced and oxidized MT differs. Previously prepared apo-MT was oxidized with 0.5 and 1 μM $K_2Cr_2O_7$ and $KMnO_4$ at 37 °C for 2 h. Then the oxidized apo-MT interacted with 66 mM Zn for 1 h at 37 °C and the absorbance was measured in intervals of 2 min. Similar to reduced apo-MT, MT aggregates with molecular mass from 20 to 30 kDa were observed by SDS-PAGE (Fig. 3A). Using the chip capillary electrophoresis allowed us to quantify MT aggregation as change in ratio of monomeric MT to total signal similarly to reduced apo-MT (Fig. 3B). The average area of 15 kDa peak was

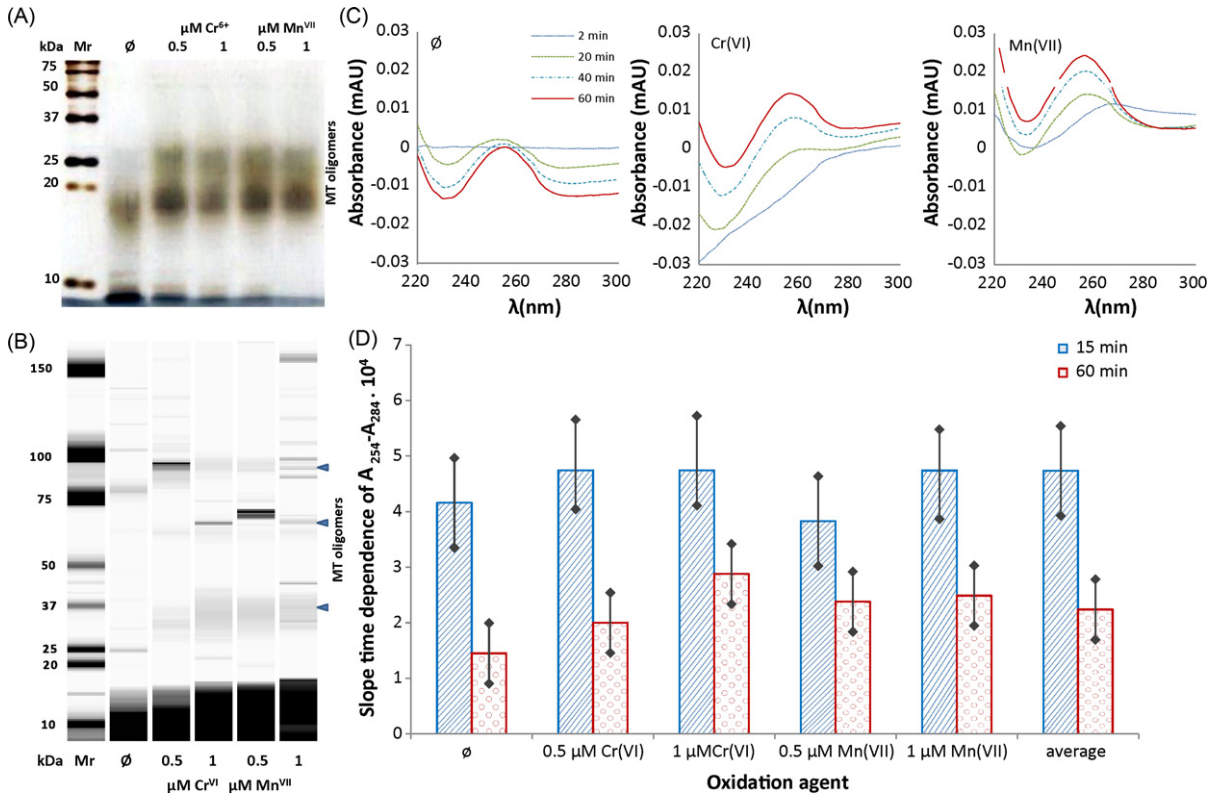

Fig. 3. Interaction of 0.03 mM oxidized apo-MT with zinc. (A) SDS-PAGE of 800 ng oxidized apo-MT after 60 min interaction with 66 mM Zn(II), in line labelled as ø water instead oxidation agent was added. (B) Virtual gel output from Experion system, 800 ng oxidized apo-MT after 60 min interaction with 66 mM Zn(II), arrows indicate MT oligomers, in line labelled as ø water instead oxidation agent was added. (C) Absorption spectra of 0.03 mM oxidized apo-MT with 66 and 100 mM Zn(II), apo-MT was oxidized with Cr(VI), Mn(VII), ø means control, at which water instead oxidation agent was added. (D) Slopes of $A_{254} - A_{284}$ time dependence (dA/dt) according to oxidation agent used and their average values, hatched bar: within the range 0 to 15 min, dotted bar: within the range from 15 to 60 min.

0.2497, which was 30% of value counted for 66 mM Zn(II) from exponential dependence for reduced apo-MT (Fig. 2B). Average ratio of 15 kDa peak to total signal was 0.0234, which was 70% of value counted from exponential dependence for reduced MT (inset in Fig. 2B). Lower peak area together with lower ratio of the peak to total signal indicated higher aggregates formation for oxidized MT. Zn(II) binding into oxidized apo-MT was also monitored spectrophotometrically (Fig. 3C). Similarly to reduced MT, we observed an increase of the absorbance within the range from 212 to 284 nm with maximum at 254 nm, which is typical for heavy-metal binding into the MT structure. Compared to reduced MT, a decrease in absorbance with minimum at 228 nm, this is probably connected to oxidation of thiol moieties [13]. After the evaluation of Zn(II) binding into MT structure we found that the average value of the 0–15 min slopes was $4.84 \times 10^{-4} \pm 8.06 \times 10^{-5}$ and the average value of the slopes within the interval from 15 to 60 min was $2.24 \times 10^{-4} \pm 5.42 \times 10^{-5}$. This is of 30% lower than for reduced apo-MT in the first interval (0–15 min) and 50% lower in the second interval (16–60 min) (Fig. 3D). Based on the statistic evaluation of data obtained, we found that oxidized apo-MT signals significantly differed from both reduced and from those obtained for 0 mM Zn(II). This indicates that Zn(II) binding into oxidized apo-MT structure is slower and the observed zinc-dependent MT aggregation occurs by a mechanism different from a simple oxidation. For biological function of MT it can be concluded that zinc concentration in the cell can be regulated by MT depending on its redox state and that oxidation-independent aggregation is one of the possible mechanisms of enhancing MT's binding capacity for Zn(II). Similar results were obtained by Hong et al. [48].

Apo-MT has now been identified in a variety of tumours and tumour cell lines [50]. Krepkyi et al. found that apo-MT can effectively cooperate with Cr(VI) in reactions damaging DNA [51]. It was found that Cr(VI) is reduced on Cr(V) by MT, which generates hydroxyl radicals [47]. These radicals can further induce oligomerization of MT [11]. As well as chromium, manganese can also induce reactive oxygen species. Affecting of MT by manganese results in the oligomerization of these proteins most likely by similar mechanism as in case of H_2O_2 [47]. Decreased rate of Zn(II) binding into MT may be associated with formation of covalent bonds between –SH moieties, which are not able to bind a metal ion.

Our results show that releasing and binding of metals by MT structure is dependent on physical conditions, which can be used to control the cell processes. Several authors report on the evidence of metalloenzymes (de)activation by zinc supply from MT molecule [52–54]. It was demonstrated that the exposure of monomeric Cd_7-metallothionein to Cd(II) ions in potassium phosphate buffer results in the non-oxidative formation of MT dimers containing approximately two additional Cd(II) ions/monomer subunit [31,55]. Meiyi et al. removed Cd and Zn metal ions from the native rabbit-liver metallothionein upon unfolding and obtained Cu-modified metallothioneins (Cu–MTs). They found that Cu(II)–MT resembles that of the native MT, whereas Cu(I)–MT forms oligomers with a higher copper content [56]. Zhong et al. studied redox process following a substitution reaction between platinum(IV) complex K_2PtCl_6 and rabbit-liver native Cd, Zn–MT. The reaction generated monomeric and dimeric products, and higher oligomers precipitate with intra- or intra- and intermolecular cys-cys linkages. Pt(IV) is reduced to Pt(II), which then binds to the monomeric and dimeric products, and may also bind to higher oligomers. The authors discussed the mechanism of the antitumour activity and developing drug resistance of Pt(IV) complex drugs [57]. Oxidative and non-oxidative induced oligomerization of MT due to zinc(II)

binding was also observed using of size exclusion chromatography with electrospray ionization mass spectrometry [58]. However, kinetics of this reaction and even employment of capillary electrophoresis have not been done yet.

4. Conclusions

According to the most recently published papers metallothionein is involved not only in storage and detoxification of heavy metals, but it has also a regulation function. The mechanisms of regulation of cellular function by MT are still not clear. Regulation function of MT can be connected to its thiol groups, redox state and binding/releasing of metal ions. Due to MT's function as an antioxidant, it can be assumed that increased level of oxidative stress can result in change of intracellular or extracellular zinc concentration and therefore to changed activity of zinc-dependent enzymes. In this study it was shown that zinc binding into MT structure and MT aggregation are dependent on redox conditions. These results suggest on the mechanism how MT can influence the regulation of cell processes.

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