Int. J. Electrochem. Sci., 7 **(2012)** 12415 - 12431

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

Review Paper

Structure, Polymorphisms and Electrochemistry of Mammalian Metallothioneins – A Review

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Received: 25 September 2012 / *Accepted:* 10 October 2012 / *Published:* 1 December 2012

Mammalian metallothioneins (MTs) are cysteine-rich low-molecular mass proteins with numerous functions including toxic metal detoxification, as a metal chaperone and maintenance of metal ion homeostasis. Four major isoforms (MT-1 through MT-4) have been identified in mammals. In this review, the functions of the isoforms are summarized. Moreover, polymorphisms of MTs genes and their connection with cancerogenesis and cancer diagnostics are described. The interesting properties of MTs are closely related with structure, which can be studied by modern bioinformatics approaches and electrochemical methods. The advantages of the mentioned approaches and methods are discussed.

Keywords: Metallothionein; Isoforms; Bioinformatics; Data Analysis; Phylogeny; DNA Sequencing; Electrochemistry; Voltammetry; Brdicka Reaction; Peak H

1. INTRODUCTION

Metallothioneins (MTs) belong to the family of cysteine-rich low-molecular mass proteins, which have been found in bacteria, plants, invertebrates and vertebrates. Mammalian MTs, a family of

non-enzymatic proteins containing 61-68 amino acids with low molecular mass (app. 6-7 kDa), which were discovered in 1957 as a cadmium-binding protein when Margoshes and Valee isolated them from horse renal cortex tissue. They have distinctive amino acid composition – high cysteine content (up to 30 %, 20 cysteine moieties in mammals) and no or very low content of histidine, phenylalanine, tyrosine and tryptophan, which means that they are rich in sulphur and metals bound in the thiolate complex. Cysteine residues form series of distinctive motifs, especially Cys-X-Cys, Cys-X-Cys-Cys, and Cys-X-X-Cys, where X represents other amino acid than cysteine. MTs have been implicated in a number of functions including toxic metal detoxification, as a metal chaperone and maintenance of metal ion homeostasis [1]. The ability to bind diverse range of monovalent, divalent and trivalent metal ions has been revealed and shown on the following ions Ag(I), Au(I), Bi(III), Cd(II), Co(II), Cu(I), Fe(II, III), Hg(II), Pb(II), Pt(II) and Zn(II). Human MTs are in the focus of interest of many scientists due to their pathological roles in diseases including tumour, cardiovascular or neurodegenerative diseases [2-5]. The genes for MTs are localized on the chromosome 16q13 in human. Classification of MTs is still complicated and there are different insights into their classification. Historically, Fowler et al. established three classes of metallothioneins. Class I comprising all proteinaceous MT with locations of cysteine closely related to those in mammals ("MTs showing homology with horse MT"). Some molluscs and crustacean MT belonged to this class, such as those characterized in mussels, oysters, crabs and lobsters. Class II including proteinaceous MT lacks this close similarity to mammalian MTs ("rest of MTs with no homology with horse MT"), while Class III consisting of nonproteinaceous MTs, into which some authors included plant cysteine-rich heavy-metal-binding peptides called phytochelatins. Complex classification system introduced by Binz and Kägi involves families, subfamilies, subgroups and isoforms. On the basis of this system, fifteen families include vertebrate, mollusc, crustacean, echinodermata, diptera, nematode, ciliate, fungi-I, fungi-II, fungi-III, fungi-IV, fungi-V, fungi-VI, prokaryotes and plants has been established. On the other hand, there is an effort to classify MTs in accordance with their metal-binding features [6].

2. MAMMALIANS' METALLOTHIONEINS

Four major isoforms (MT-1 through MT-4) have been identified in mammals. In addition, at least thirteen known closely related MT proteins in humans have been described. MT-1 and MT-2 are the most widely distributed MT isoforms. They are expressed in many cell types in different tissues and organs with the highest expression in liver and kidney. Their ability to bind metal ions is well known. On the other hand, some MT1 sub-isoforms as well as MT-2 are connected with diseases. This fact is closely connected with the wide range of MT-1/2 inductors, such as reactive oxygen and nitrogen species (ROS and RNS, respectively), glucocorticoides, proinflammatory cytokines or catecholamines. Generally, MTs-1/2 play important role in the metal homeostasis, ROS and RNS scavenging, immune defense responses, angiogenesis, cell cycle regulation and progression, cell differentiation and regulation of zinc-containing proteins and zinc fingers. The role of MT-1/2 in brain injury is still discussed. It has recently been revealed that *MT-1/2* expression is induced in the liver after brain injury and cause zinc sequestration [7]. Compared to MT-1 and MT-2, MT-3 and MT-4

demonstrate very limited cell-specific pattern of expression. MT-3 represents a unique metalloprotein called also neuronal growth inhibitory factor (GIF) because of the ability to inhibit outgrowth of neuronal cells [8,9]. Its function is connected with c-Abl protein activation via epidermal growth factor (EGF) receptor signalling with subsequent modulation of cytoskeleton in astrocytes [10]. Initially, it has been isolated from the human brain with a metal content of four Cu(I) and three Zn(II) ions per one MT-3 molecule. However, Cu(I) binding capacity of MT-3 is discussed and is questionable in the *in vivo* conditions. MT-3 has also been obtained from bovine and equine brain and from mice with non-functional *MT-1* and *MT-2* genes. This fact shows the ability of MT-3 to bind heavy metal ions. It has been reported to be downregulated in the Alzheimer´s disease. In this case, MT-3 is able to bind Cu(I) when Cu homeostasis is disrupted and Cu together with Zn ions ate implemented in the formation amyloids by modulating the aggregation of amyloid-beta peptides [11]. The aggregation of alpha-synuclein (alpha-Syn), the major component of intracellular Lewy body inclusions in dopaminergic neurons of the substantia nigra, plays a critical role in the etiology of Parkinson disease (PD). This process is closely connected with the misbalance of Cu ions. Meloni and Vasak established that alpha-Syn-Cu(II) possesses catalytic oxidase activity, thus, it can promote the production of hydroxyl radicals, alpha-Syn oxidation and oligomerization. Zn7MT-3, through Cu(II) removal from the alpha-Syn-Cu(II) complex, efficiently prevents its deleterious redox activity [12]. The role of MT-3 in cancerogenesis and progression of glial tumours and ependymomas is discussed [13,14]. In the light of the role of zinc ions in the induction of caspase-3, MT-3 prevents oxidative stress via Zn/Cu homeostasis and has significant neuroprotective effect [15,16]. MT-3 can also probably regulate psychological behaviour. This phenomenon has been demonstrated on MT-3 deficient mice, where MT-3 knock-out mice had significantly shorter social interactions [17]. Medical consequences must be further discussed. MT-3 has been found also in the male reproductive organs and a plenty of various tissues [18,19]. MT-3 mRNA was detected in the cerebrum, the dorsolateral lobe of the prostate, testis, and tongue of Wistar rats. Immunohistochemistry revealed the presence of MT-3 in some cells of the glomerulus and the collective tubules in the kidney, some cells in the glandular epithelium of the dorsolateral lobe of the prostate, some Sertoli cells and Lydig cells in the testis, and taste bud cells in the tongue [19]. In comparison with MT-1 and MT-2, MT-3 shows distinct chemical, structural and biological properties. MT-4 belongs to noninducible proteins, with its expression primarily confined to squamous epithelia. Its presence is restricted to stratified squamous epithelium, a tissue providing a protective surface on skin, footpath, tail, tongue, the upper part of the alimentary tract, and the vagina of rodents. In addition, MT-4 expression in maternal deciduum together with the expression of entire MT gene locus have been reported in mouse [20]. It plays crucial role in the regulation of zinc and copper homeostasis. In addition, a gene called *MT-like 5* (*MTL-5*) that encodes a testis-specific cysteine-rich MT-like protein called tesmin was described in the q13 region of chromosome 11. Tesmin plays a specific role in both male and female meiotic prophasis I [21]. Tesmin undergoes very dynamic localisation, which suggests that it plays crucial role in multiple stages of spermatogenesis and spermiogenesis, possibly during sperm maturation and/or morphogenesis [22].

The role of MT in cells is not fully understood, especially with respect the MT involvement in the regulation of cellular processes. MT induces expression of genes of protective and anti-inflammatory factors, such as IL-10, fibroblast growth factor (FGF), transforming growth factor-beta

(TGF-□), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), neurotrophin (NT), and their receptors. These factors are involved in the mediating repair, angiogenesis, and vascular remodelling [23]. On of the best model that can represent role of MTs in gene expression is keloid fibroblast, cell with the high proliferative capacity compared to normal fibroblast. The down-regulation of the MT-2A gene in proliferating keloid fibroblast by siRNA-mediated silencing enhanced cell proliferation with concomitant up-regulation of the NF-kappa B gene and 10 of 13 other NF-kappa B pathway-related genes [24]. These changes were closely connected with the enhanced keloidogenesis with possible involvement of the NF-kappa B signalling pathway. Interleukin-10 (IL-10), transforming growth factor-beta (TGF-β) and vascular endothelial growth factor (VEGF) genes belongs to the group of the most important genes of which overexpression is induced by MTs. Zinc fingers are the small structural motifs that are characterized by the coordination of one or more zinc ions in order to stabilize fold. They regulate gene expression. In this view, zinc exchange between MTs and zinc fingers regulates gene expression and involvement of MTs is well evident. However, the number of works focused on these questions is still limited [25-27].

3. MTS GENES AND POLYMORPHISMS

MTs genes are tightly linked, and at a minimum they consist of eleven MT-1 genes (MT-1A, -B, -E, -F, -G, -H, -I, -J, -K, -L, and -X), and one gene for each of the other MTs isoforms (the MT-2 A gene, MT-3 gene, and MT-4 gene). Expression of MTs is started by binding of metal regulatory-transcription factor – 1 (MTF-1) to the regulative region of MTs gene called metal responsive element (MRE). The nomenclature for MTs isoforms has not been standardized until now. MTs genes are intensely studied not only in the association with heavy metal ions exposure, but also in the connection with the pathogenesis of various diseases, such as processes of chronic inflammation and malignant diseases, and ageing in human [28]. In this light, polymorphisms of MT genes that are present within the human population are in the focus of interest, especially due to possible health implications. Role of *MT-1A* polymorphism was studied in elderly women in Italy. The authors found that *+647 A/C MT-1A* polymorphism corresponding to A/C (Asp/Thr) transition at 647 nt position rather than *+1245 MT1A* (A/G – Lys/Arg transition at 1245 nt position) is connected with higher zinc release by MT, low MT levels and reduced IL-6 plasma concentrations resulting in lower inflammatory status [29]. On the other hand, Giacconi et al. proved that *+1245 MT1A G+* genotype is connected with higher risk of cardiovascular disease in Greece [30]. MT2A -5 G(-) carriers may be more advantageous for longevity in the Turkish population [31]. However, diet and other factors, such as disease predisposition, must be further studied. In another study, Giacconi et al. showed that *+647 A/C MTIA* polymorphism is probably connected with diabetes mellitus 2. C+ carries are associated with higher glycaemia and glycosylated haemoglobin and thus worse glycemic control. Similar results were found by Liu et al. [32]. Yang et al. used the polymerase chain reaction (PCR)-based restriction fragment length polymorphism method for the detection of seven single-nucleotide polymorphisms in MT1A, MT1B, MT1E and MT2A genes and possible risk of type 2 diabetes mellitus and its complication in 851 Chinese people [33]. Their results suggest that multiple single nucleotide polymorphisms in MT genes

are associated with diabetes and its clinical symptoms. Furthermore, MT1A gene in rs8052394 single nucleotide polymorphism is most likely the predisposition gene locus for diabetes or changes of serum superoxide dismutase activity. Superoxide dismutase together with MTs has significant protective effect on cells against oxidative damage [34]. These facts indicate possible involvement of MT suggesting a possible role of MTs polymorphisms in diabetes mellitus and its cardiovascular complications [35,36].

ROS are assumed to be involved in the pathogenesis of many diseases including amyotrophic lateral sclerosis. MTs are able to scavenge ROS, thus, their involvement in ROS-mediated diseases may be considered, especially in the light of the possible single-nucleotide polymorphism and changes in MTs transcription induction [37]. The second mechanism of the involvement of MT in pathogenesis is based on the regulation of zinc(II) ions that affect zinc-regulated gene expression. This fact was shown by Mazzatti et al. in the study focused on the zinc-dependent transcription of pro-inflammatory cytokines and alterations in metabolic regulatory pathways [38] or by Bellomo et al., who demonstrated regulation of cytosolic zinc levels, ZiP and MT gene expression by glucose in primary pancreatic islet beta cells [39]. However, these questions are very complex with many consequences. Gundacker et al. investigated polymorphism in glutathione-S-transferase GSTT1 and GSTM1 genes and possible correlation with mercury concentrations in body fluids and hairs and gene expression of *MT1* and *MT3*. Results of this study indicate that GSTT1 and GSTM1 deletion polymorphism is a risk factor for the increased susceptibility to mercury exposure. In addition, MT1X expression was significantly higher in person with intact GSTT1 and GSTM1 genome [40]. Downregulation of MTs transcription may result in the changes in antioxidant mechanisms and may contribute to pathogenesis of many diseases. In the case of amyotrophic lateral sclerosis, the single-nucleotide polymorphism of the MT2A and MT3 genes is not associated with the sporadic amyotrophic lateral sclerosis in a Japanese population [41]. Polymorphisms in MT2A gene are also studied in the connection with atherosclerosis [42] and especially with heavy metal ions exposure by Cd, Pb, Zn and Cu levels [31,43-48]. Similar results have been demonstrated for MT-4, where workers with MT4-216 A/G genotypes exposed to Pb for extend period of time were analysed. Analysis revealed that workers with G allele were more susceptible to the toxic effect of Pb compared to those with AA type allele [49]. Role of MTs in malignant diseases is widely discussed especially due to changes in the expression of some MTs in tumour tissues. Role of MTs genes polymorphisms in malignant processes may be assumed. Forma et al. examined A/G (-5) polymorphism in the promoter region of the metallothionein 2A gene (MT-2A) in ductal carcinoma of the breast, however, their results suggest that the A/G (-5) polymorphism in the promoter region of the MT-2A gene may not be linked with neoplastic transformation of breast ductal carcinoma [50]. Risk of oral squamous cells carcinoma is significantly reduced in MT-1 rs11076161 AA, rs964372 CC, and rs7191779 GC genotypes, whereas individuals carrying the MT-1 rs8052394 A allele seem to be exposed to higher risk of this type of malignant disease [51]. It seems that not only MT, but also polymorphisms in MT genes plays very important role in the processes of cancerogenesis and progression of malignant disease.

4. MTS, CANCEROGENESIS AND CANCER DIAGNOSTICS

Table 1. Expression of MT isoforms in tumour cell lines and tumour tissues.

MT isoform	Cell line/Tissue	Significance	Implication/note	Refs.
MT-1E	Increased methylation in some malignant tissues (malignant melanoma)	Regulation of cell proliferation *MT-1E* – possible tumour suppressor gene	Increase the sensitivity of malignant cells to cisplatin	[55]
	Increased methylation in the endometrial carcinoma cells	Regulation of cell proliferation *MT-1E* – possible tumour suppressor gene	Coincident low oestrogen receptor alpha expression	[56]
	Malignant glioma cells	Regulation of migration and invasion of tumour cells	Metastatic process	[57]
	Human bladder cancer cells	Migration, invasion and progression of tumour	MT-1E necessary for cell migration together with nicotinamide N-methyltransferase	[58]
	oestrogen receptor-negative human invasive ductal breast cancer	Overexpression of *MT-1E* gene	Possible specific functional role mediated via effector genes downstream of the oestrogen receptor	[59]
MT-1F	Colon cancer tissue	Downregulation of *MT-1F* expression connected with the loss of heterozygosity	Putative oncosuppressor	[60]
	Breast carcinoma cells	Higher *MT-1F* expression in grade 3 malignant tumours	Implication in cell differentiation	[61]
MT-1G	Human hepatocellular carcinoma	Tumour suppressor gene	Regulation of promoter hypermethylation	[62]
	Papillary thyroid carcinoma	Downregulation of *MT-1G*. Oncosuppressor.	Affecting of cell growth and proliferation	[63]
MT-1H	Human acute myeloid leukaemia	Aberrant MT-1H promoter methylation	Response to AML treatment	[64]
	noninvasive MCF7 breast cancer cells, highly aggressive NEDA-MB-231 breast cancer cells, breast myoepithelial cells	variant MT-1H isoform with changes in amino acid residues in the protein sequence	differences in the predicted secondary protein structure	[65]
MT-1X	Urinary bladder cancer cells	Overexpression of *MT-1X* independently on the tumour grade	Role of MT-1X in the urinary bladder cancer cells – no change in other *MTs*	[66]
MT-2A	MDA-MB-231 breast cancer cell line	*MT-2A* overexpression increases *matrix metalloproteinase-9* expression	Higher invasivity	[67]
	anchorage-dependent MCF-7 cell line	*MT-2A* downregulation leads to entosis	Higher invasivity	[68]
	MCF-7 cell line	*MT-2A* expression leads to the progression from G1-to S-phase and regulation of cdc25A signalling	Cell proliferation	[69]
	HELa cell line	Induction of *MT-2A* by rotenone	Protection against ROS	[70]
	human invasive ductal breast carcinoma tissue samples	Higher *MT-2A* mRNA transcript level, connection with grade 3 tumours	Cell proliferation	[71]
MT3	Human adipocytes; human (obese) subcutaneous and omental adipose tissue	Hypoxia-induced *MT-3* expression	Adipocyte protection against oxidative damage	[72]
	Aesophagial carcinoma cell lines OE33 and FLO-1	*MT-3* silencing due to DNA hypermethylation and histone changes	Progression in cell growth and proliferation	[73]

Since the 1980´s, it has been established that MT plays important role in the processes of cancerogenesis and represents not only a predictive prognostic factor in the human malignancies, but also a predictive factor in the resistance to anti-tumour therapy. Changed MT-1/2 mRNA and/or protein levels have been found in many tumour cell lines and tumour tissues, which included not only solid tumours, but also haematological malignancies. Expression of MT isoforms in different types of tissues, mainly tumour, is summarized in Tab. 1. Decreased downregulation of MT-1 isoforms has been determined in some tumour tissues (central nervous system, hepatic, prostate, thyroid). This downregulation is probably connected with the hypermethylation of the MT promoter. 50 – 100% repression of *MT-1/2* expression in primary hepatocellular carcinoma based on the activation of phosphatidylinositol 3-kinase (PI3K)/AKT pathway inducing dephosphorylation of the transcription factor CCAAT/enhancer-binding protein(C/EBP)-alpha was observed [52]. On the other hand, MTs enhanced expression was shown in breast, kidney, lung, nasopharynx, ovary, salivary glands, testes and urinary bladder as well as in leukaemia and non-Hodgkin´s lymphoma. However, changes in *MT-1/2* expression are tumour specific and must be discussed individually. Arriaga et al. immunohistochemically evaluated MT-1/2 isoforms in the colorectal tumours. Whereas isoforms (MT1G, 1E, 1F, 1H, and 1M) were lost during the transition from normal mucosa to tumour, MT1X and MT2A were less down-regulated. Authors also demonstrated that a lower immunohistochemical expression was associated with poorer survival of patients. [53]. Expression of MT genes may be involved in the resistance to antitumor therapy. This fact is confirmed by Yang et Chitambar, who demonstrated expression of MT-2A gene as a response to treatment by antineoplastic agent gallium nitrate in CCRF-CEM cells [54].

5. MT STRUCTURE AND BIOINFORMATICS APPROACHES FOR STUDYING AND CLASSIFYING OF MT STRUCTURE

5.1. MTs primary structure

Data about primary structure of MTS are known only for the limited number of species. Generally, these proteins are called “cysteine rich”, which means that primary structure is rich in cysteine. In addition, aromatic amino acids do not occur in these proteins. Cysteine residues are the most highly conserved followed by the basic amino acids lysine and arginine. The metal binding domain of MT consists of 20 cysteine residues juxtaposed with Lys and Arg arranged in two thiol-rich sites called a and b. Twenty cysteine residues occur in primary sequence in following repetitions: Cys-X-Cys, Cys-Cys-X-Cys-Cys, Cys-X-Cys-Cys, where X is amino acid different from cysteine. The study of Huang et al. was among the first papers focused on the primary MT structure. They studied MT isolated from the mouse liver [74]. Since then, papers focused on the characterization of MT primary structure have been published [75-83]. The representation of mammalian MTs is introduced in Tab. 2, signatures with the common motif with vertebrates is presented in Fig. 1.

Table 2. Summary of the common motives for the mammalian MT1-4 isoforms presented in the PROSITE format. Motif identical to PS00203 (Homo sapiens) is blue labelled. Data were obtained from the Uniprot database.

Isoform	Regex	Score	AA	Seqs	Ident
MT1	C-S-C-x-[APT]-[DGSV]-x-[ST]-C-[AST]-C-[AST]-x-[ST]-[CS]-x-[CS]-x(3)-[KR]-x-[APST]-S-C-K-x-[CNS]-C-C-[AS]-C-C-P-x-[DGS]-C-[AST]-[KR]-C-A-x-G-C-x-C-K-[EG]-[ASTV]	131,4970	48 (5-53)	54	Min. 76%
MT2	S-C-[AST]-C-[APS]-[GNS]-[AS]-C-x-C-K-[ADEQ]-C-[KR]-C-[AT]-[ST]-C-K-K-S-C-C-S-C-C-P-[APV]-G-C-[AT]-[KR]-C-[AS]-Q-G-C-[IV]-C-K-[EG]-A-x-[DE]-K-[CG]-[NS]-C-C-A	180,6087	49 (12-61)	25	Min 80%
MT3	D-P-E-[APST]-C-P-C-P-[AST]-G-G-S-C-T-C-[ADES]-[DG]-x-C-K-C-x-G-C-x-C-[AT]-[ADNS]-[CS]-K-x-S-C-C-S-C-C-P-A-[DEG]-C-x-K-C-[AT]-K-D-C-V-C	174,9267	49 (2-51)	23	Min 83%
MT4	C-[STV]-C-[LM]-S-[EG]-G-x-C-x-C-G-D-N-C-K-C-T-[NST]-C-[NS]-C-x(4)-K-S-C-C-[AP]-C-C-P-P-G-C-A-K-C-A-[QR]-G-C-[IV]-C-K-x-[AGV]-[AS]	168,2312	49 (6-55)	23	Min 90%
seqs – number of sequences, ident. – minimal similarity between sequences, AA – length of motif (position of motif), score - the correspondence motif in the sequences					

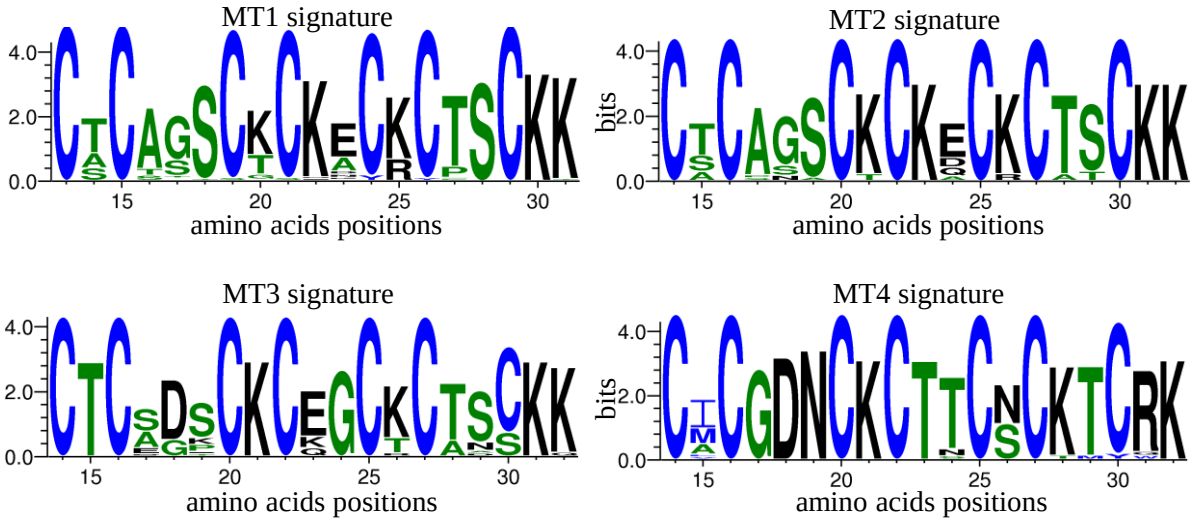

Figure 1. MT1-4 signatures presented for the common motif with vertebrates (PS00203).

5.2. MTs secondary structure

The secondary structure of MTs is still poorly understood. In addition, functional significance of MTs secondary structure is not evaluated. Lueber and Reiher investigated secondary structure of the β domain of rat metallothionein. α turns were the only established secondary structure elements occurring in the molecule [84]. The prediction of MTs secondary structure is shown in Fig. 2.

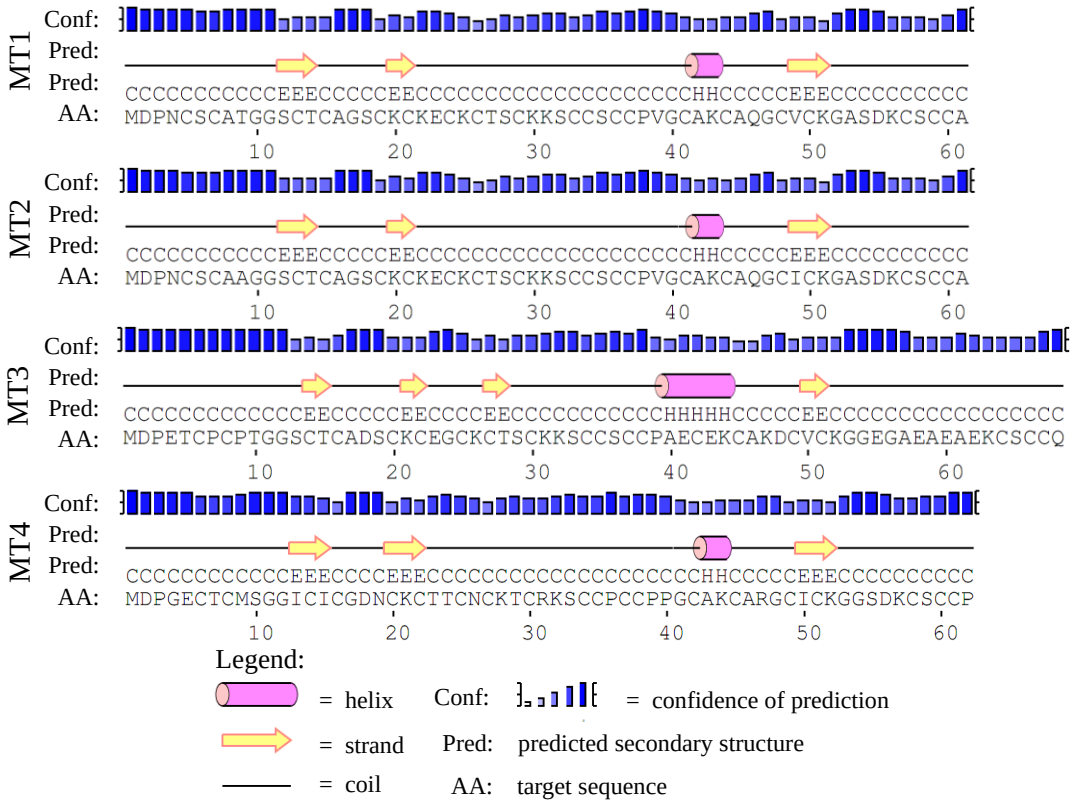

Figure 2. Prediction of the secondary structure of MTs for MT1-4 isoforms. Prediction was carried out using the PSIPRED 3.0 and "feed-forward" method based on the neuronal networks.

5.3. MTs tertiary structure

MTs tertiary structure is highly variable and depends on the bounded metal ion(s). Divalent metal ions bonded to sulfhydryl groups of cysteine moieties form tetraedric configuration of thiolate clusters. MT demonstrates the highest affinity for Pt(II) (stability constant 10^{24} – 10^{22}), Cu(I) (10^{19} – 10^{17}) followed by Cd(II) (10^{17} – 10^{15}), and Zn(II) (10^{14} – 10^{11}). However, metal complexes of MTs may be affected by many factors, such as reductive radical stress, which leads to the desulphurization reactions involving cysteine moieties [85,86]. MT tertiary structure consists of two separate domains: C-terminal α-domain (amino acids 31-61) and N-terminal β-domain (amino acids 1-30), linked by a short bridging region (Fig. 3). α-domain contains 11 cysteine moieties that can bind four divalent or six monovalent ions under the formation of an adamantane-like structure. β-domain contains 9 cysteine moieties able to bind three divalent or six monovalent metal ions under the formation of a hexane-like

cluster. Finally, MT is able to bind 7 divalent and 12 monovalent metal ions. In the absence of metal ions, apo-thionein (apo-T) is predominantly unstructured, 3D structure is formed only upon metal coordination. Rigby et al. demonstrated the migration of the 11 cysteinyl sulphurs in the α domain and the 9 cysteinyl sulphurs in the β domain to the outside of the protein while the polypeptide backbone adopted a random coil conformation. This cysteinyl sulphur inversion is necessary for metal scavenging in the surrounding environment under the formation of the more stable and proteolytically protected metal-bound MT [87]. The model of the mammalian MTs with marked cysteine moieties is shown in Fig. 4.

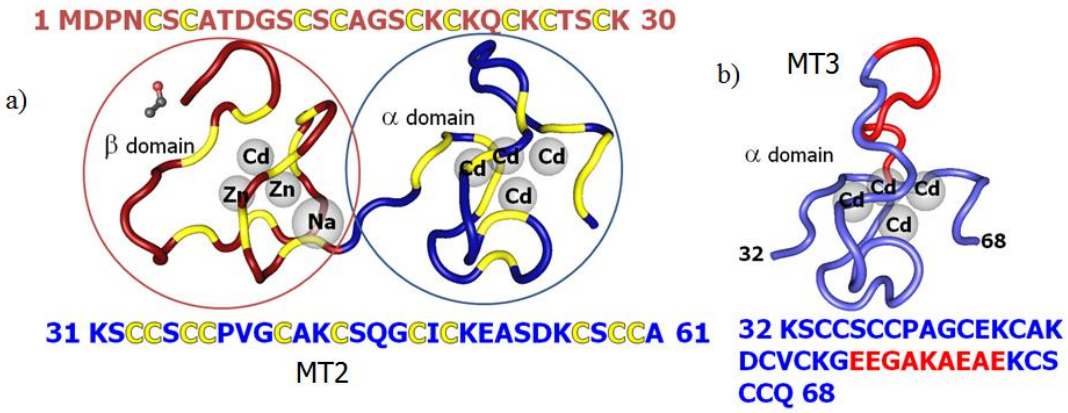

Figure 3. a) Tertiary structure of Rattus norvegicus MT with the metal ions. Domains are blue marked (α domain) and red marked (β domain). Cysteine moieties are yellow marked. b) Molecular structure of the MT-3 isolated from *Mus musculus*. Well evident insertion in the primary structure is redly highlighted.

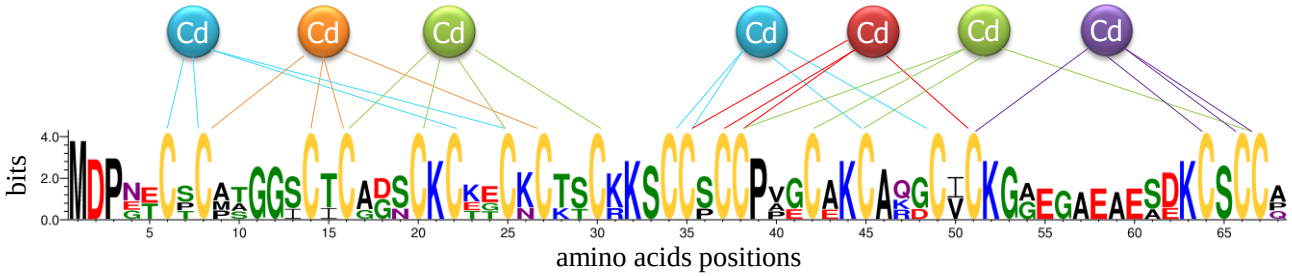

Figure 4. Signatures for complete sequences of mammalian MTs. 125 MT sequences were used for the proposition of this model. Cysteine moieties are yellow marked. MTs metal-binding properties are well-evident.

In spite of many studies focused on the MTs, there is only limited number of information about the origin, evolution and diversification of MTs. These questions may be revealed by the bioinformatic approach. Database Uniprot (http://www.uniprot.org) serves as a source of the primary structure of mammalian MTs. This database may be used for the comparison study of MTs.

6. ELECTROCHEMICAL DETERMINATION AND STRUCTURAL STUDYING OF MTS

Techniques used for MTs determination are summarized in the review of Ryvolova et al. [88] and Adam et al. [89]. From those, electrochemical methods have enabled us to lower the limit of detection to 10^{-18} M and significantly increase the sensitivity [90]. Electrochemical methods take the advantage electroactivity of sulphydryl groups, which can undergo oxidation, or may catalyse evolution of hydrogen from the supporting electrolyte. The increase of sensitivity has been achieved by the proposition and improvement of the adsorptive transfer stripping technique, where MT is accumulated on the surface of a hanging mercury drop electrode and interfering compounds are rinsed out [90-94]. Voltammetric methods useful in the MT analysis include linear sweep, cyclic, differential pulse and square wave voltammetry (Tab. 3). The special focus is devoted to the Brdicka reaction and H peak.

Table 3. The most important electroanalytical methods in MT analysis.

Method	Application	Refs.
Anodic stripping voltammetry	MT determination, MT binding studies	[95-99]
Cathodic stripping voltammetry	MT interaction studies, MT determination	[90,100-103]
Cyclic voltammetry	MT determination, MT and MT-related peptide interaction studies	[90,104-106]
Differential pulse polarography	MT determination in tumour cell lines, MT in biomonitoring. MT binding properties.	[90,107-110]
Differential pulse voltammetry	MT interaction studies	[92,111]
Linear sweep voltammetry	MT binding studies	[112]
Square wave voltammetry	MT determination, MT antioxidant properties, MT binding studies	[100,101,113]

The method of the polarographic determination of the proteins with sulphhydryl groups in ammonia buffered cobalt(III) solution was originally described by Rudolf Brdicka in 1933 [114]. The method is based on the catalytic evolution of hydrogen (catalytic hydrogen signal – Cat) on a dropping mercury electrode from the solutions of thiols and disulphides in the presence of cobalt(III) salt. Since the 1933, when the method was originally described, it has been used for the study of blood proteins [115] or bilirubin [116]. Brdicka methods underwent some improvements, such as the application of more selective and specific pulse techniques and the static mercury electrode (hanging mercury drop electrode). These modifications are known as a modified Brdicka reaction. Kolthoff was one of the firsts who studied behaviour of cysteine, cysteine ethyl ester and cysteamine using the Brdicka reaction [117]. Polarographic Brdicka activity of cysteine was studied also by Mader and Vesela [118]. Modification of Brdicka reaction led to its more common application especially in the environmental and medicinal studies. Raspor et al. were amongst the firsts who used modified Brdicka reaction for

the determination of MTs [119]. Nowadays, modified Brdicka reaction is used for determination of metallothionein in blood from patients suffering from malignant tumour disease [5,93,120-128], or for biomonitoring of environmental pollution and ecological studies [97,124,129-139]. In addition, Brdicka-like reactions, it means reactions based on the Brdicka principle, are still investigated. Selesovska-Fadrna et al. studied cysteine and cysteine-containing peptides glutathione, gamma-Glu-Cys-Gly and phytochelatin $(\gamma\text{-Glu-Cys})_{(3)}$-Gly (PC3) in the presence of Co(II) ions using a silver solid amalgam electrode (AgSAE) with differential pulse voltammetric (DPV) technique. The sensitivity of 10^{-8} M has been reached [140].

The peak H (because of High sensitivity, Hydrogen evolution and Heyrovsky) technique has been established and described by Mader et al. as a method based on the catalytic evolution of hydrogen in the presence of a protein [141]. H peak differs from the polarographic and voltammetric catalytic signals of peptides by its ability to detect peptides and proteins down to nanomolar and subnanomolar concentrations and by its high sensitivity to local and global changes in protein structure [142]. The character and origin of the catalytic peak H is not clear yet. Free –SH moieties together with $–NH_2$ ones are involved in the catalysis of hydrogen evolution at very negative potentials [88]. No metal ions in the supporting electrolyte are needed. pH of the supporting electrolyte (usually borate buffer, pH = 8) together with content of oxygen are the most important factors in the MT analysis. MT provides a signal at a potential about -1.7 V [143]. Constant current stripping chronopotentiometry was used by Kizek et al. for the determination of metallothionein from rabbit liver in subnanomolar concentrations [143]. A coupling of derivative chronopotentiometry with adsorptive transfer stripping technique on a hanging mercury drop electrode enabled determination of MT [137,144]. This method has found its position in the biomonitoring and medicine for the determination of MT in the tissues of wild perch in connection with exposure to heavy metals [97], or in the other study focused on in the tissue samples of perches and their parasites [145].

Flow injection analysis with electrochemical detection was shown as suitable for determination electrochemical profile of interaction between 23 sulphur-rich fragments of the metal-binding protein metallothionein and cisplatin was studied. To evaluate the results, interaction constants were suggested. Here, we found that the maximum increased interaction (more than 100 %) occurred, when conservative aminoacids were substituted for more than one position outside the cysteine cluster. On the contrary, amino acid substitution within the cysteine cluster led to a reduction in interaction constants (up to 10-25% of average). This result clearly indicates that aminoacids outside cysteine binding motif are of high importance for interactions of metallothionein with cisplatin [146].

4. CONCLUSIONS

It is clear that metallothioneins can be considered as multitasking proteins interesting for biochemistry, clinical chemistry, and also analytical chemistry. Due to their unique primary structure (no aromatic aminoacids, rich in cysteine moieties) these proteins are involved in many biochemical pathways including scavenging of reactive oxygen species, detoxifying of various xenobiotics and metal ions, transporting of essential metal ions, cell proliferation, which most probably makes them

important for tumourogenesis. MTs can also play important role in chemoresitance to the platinum-based cytostatics and probably also to some "non-metal cytostatic drugs". Therefore MT detection in tumours may be used as a predictive marker. For these purposes, electrochemical methods are the most sensitive and can be used not only for metallothioneins quantification but also for structural studies.

ACKNOWLEDGEMENTS
Financial support from NanoBioTECell GA CR P102/11/1068 and FNUSA-ICRC CZ.1.05/1.1.00/02.0123 is highly acknowledged.

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