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Radical scavenging abilities of fish MT-A and mussel MT-10 metallothionein isoforms: An ESR study

Alessandra Buico [a], Claudio Cassino [a], Francesco Dondero [a,c], Laura Vergani [b,d], Domenico Osella [a,*]

[a] *Dipartimento di Scienze dell'Ambiente e della Vita, Università del Piemonte Orientale "A. Avogadro", Via Bellini 25/G, 15100 Alessandria, Italy*
[b] *Dipartimento di Biologia, Università di Genova, Corso Europa 26, 16132 Genova, Italy*
[c] *Ikzus Ricerche Mare e Ambiente Ltd., Via Fiasella 1, 16121 Genova, Italy*
[d] *C.I.R.B.C.MET. Center on Biology and Chemistry of Trace Metals, University of Genova, Corso Europa 26, 16132 Genova, Italy*

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Abstract

Metallothioneins (MTs) are cysteine-rich proteins involved in homeostasis of essential metals, detoxification of toxic metals and scavenging of free radicals. Scavenging of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was measured by means of ESR spectroscopy for two recombinant MTs from aquatic species: MT-10 from the sea mussel *Mytilus galloprovincialis*, and MT-A from the fish *Oncorhyncus mykiss*. Both the zinc- and the cadmium-loaded forms (Zn_7-MTs and Cd_7-MTs) were analysed, using the commercial MT-II (Zn_7-MT-II and Cd_7-MT-II, respectively) from rabbit liver as a reference. A decrease in the scavenging ability was observed for all the three MTs passing from the Zn- to the Cd-loaded forms, because of the higher stability of the Cd-mercapto complex. The Zn_7-MTs from aquatic species were more effective in scavenging DPPH signal than the rabbit Zn_7-MT-II (2.8 and 4-folds, respectively). Similar results were obtained also for the Cd_7-MTs, thus confirming the stronger antioxidant power of MTs from aquatic organisms compared with the rabbit MT-II. Moreover, mussel MT-10 was more active in DPPH scavenging than fish MT-A. When the complete release of metals from MTs was obtained by lowering the pH to 3 or, alternatively, by adding the chelating agent diethylenetriaminepentaacetic acid (DTPA), an increase in the scavenging ability of MTs was observed.

Keywords: Metallothionein; Radical scavenger; Oxidative stress; Metal release; ESR spectroscopy

1. Introduction

Metallothioneins (MTs) are small (6–7 kDa) non-catalytic proteins with high affinity for metal ions from groups 11 and 12. MTs display an unusual amino acid sequence, usually lacking histidines and aromatic residues, and having a high content of cysteine (Cys) residues. Cys represent one-third of the total amino acids and are distributed in typical motifs such as CC, CXC or CXYC sequences [1].

MTs have been found in almost all organisms, including vertebrates, invertebrates, plants and bacteria [2]. MTs are involved in crucial cellular processes such as sequestration of toxic metals (Cd, Hg), storage and release of essential metals (Zn, Cu), and scavenging of radicals [3].

MTs from vertebrates usually present 61–62 amino acid residues, whereas MTs from molluscs and nematodes have larger chains of 71–75 residues. On the contrary, MTs from insects and fungi have usually shorter chains [4]. The crystal and solution structures of the mammalian cadmium/zinc metallothioneins (i.e. the rabbit MT-I and MT-II) show twenty reduced Cys residues bound to seven divalent metal ions forming two metal-thiolate clusters. This

* Corresponding author. Tel.: +39 0131 360266; fax: +39 0131 360250.
E-mail address: domenico.osella@mfn.unipmn.it (D. Osella).

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structure is also typical of Cd_7-MTs from fish, as recently observed by [^{1}H, ^{113}Cd] NMR experiments for the Antarctic fish metallothionein MT_nc [5]. On the other hand, mussel MT-10 contains 73 amino acids with 21 Cys residues binding seven metal ions, but the 3D structure of this protein is still unknown.

Because of their pleiotropic role, MTs have been postulated to participate in many physiological and pathological processes, including cell growth and differentiation [6], tumor progression [7], and neurological disorders [8].

Reactive oxygen species (ROS) cause membrane peroxidation and DNA breaks; these processes are often associated with aging and diseases [9,10]. Numerous antioxidant systems prevent tissue damage by ROS in cells. These systems include both antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and non-enzymatic antioxidant molecules such as glutathione (GSH), metallothioneins, and ascorbic acid [11]. A lot of evidence supports the role of MTs as radical scavengers *in vitro* [12,21]. Recently, the redox activity of MTs has been observed also *in vivo* where oxidative stress increased the amount of the oxidized apoprotein (thionin) with respect to the reduced apoprotein (thionein) [13]. It has been reported that the radical scavenging activity of MTs may consist in both a direct interception of free radicals and/or in the chelation of redox active transition metal ions responsible for radical generation via the Fenton reaction [14]. In the case of Zn-loaded MTs, the redox activity mainly resides in the Cys sulfur ligands of zinc. Oxidation releases zinc, while reduction re-generates zinc binding capacity [12].

Electron spin resonance (ESR) represent a potent tool for determining the level of oxidative stress both *in vitro* and *in vivo* [15]. Usually, the antioxidant activity of a molecule is measured by evaluating its ability to scavenge oxygen radicals produced *in vitro*. Superoxide radicals are usually generated via hypoxantine/xantine oxidase system, whereas hydroxyl radicals mainly originate from a Fenton reaction [16]. Because of their short lifetimes and broad line width, both these oxygen radicals cannot be detected directly by ESR in aqueous solution at room temperature, and they require a spin-trapping agent to be measured. Nitrone spin traps are by the far the most popular ones, and among them 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) is the most used for studying oxygen-centred free radicals. Both the systems generating oxygen free radicals and the reaction of spin-trapping are strongly influenced by the chemical environment. In particular, the kind of buffer, the presence of either redox active transition metals, iron in particular, or of chelating agents can interfere with the result [17,18]. In this study, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was employed for the measurement of the antioxidant capacity of MTs. Even though this radical is not present in biological systems, it is often employed as the standard radical for antioxidant studies, since it is stable in the presence of water and air, it does not display interferences and can be directly detected by ESR [19].

This study was aimed at comparing the radical scavenging activities of two recombinant MTs from different aquatic organisms, namely MT-10 from the invertebrate *Mytilus galloprovincialis* and MT-A from the vertebrate *Oncorhyncus mykiss*. Although the two proteins show an identical cadmium content and a similar metal-binding ability, significant differences in the amino acid sequence, secondary conformation, thermal stability, and metal release have been reported for the two Cd_7-MTs [21]. It is noteworthy that the amino acid sequences of different fish MTs are rather similar to those of mammalian MTs (i.e. rabbit MT-II). For each MT, both the Zn- and the Cd-loaded forms (Zn_7-MT and Cd_7-MT, respectively) were analysed. The behaviour of Zn- and Cd-loaded MT-A and MT-10 proteins was compared with that of Zn- and Cd-MT isoform II from rabbit liver, representative for mammalian MTs, respectively.

2. Experimental

2.1. Chemicals

All chemicals, molecular weight markers and rabbit MT-I were supplied by Sigma–Aldrich (Milan, Italy). Reagents for bacterial growth were purchased from Fluka (Milan, Italy). T4 DNA ligase and Taq polymerase were supplied by Stratagene (La Jolla, CA), restriction enzymes and dNTPs by Promega Italia (Milan, Italy). Expression vector pGEX 6P-1, *Escherichia coli* strains and glutathione-sepharose 4B matrix were purchased from Amersham Biosciences (Uppsala, Sweden). Commercial Zn_7-MT-II extracted from rabbit liver was provided by Ikzus (Genova, Italy), it represents a mixture of the two isoforms MT-2a and MT-2c.

2.2. Protein metal content

The content of zinc, cadmium, copper and iron of each MT was checked by using inductively coupled plasma-mass spectrometer (ICP-MS) (X5 ICP-MS, Thermo Elemental, Winsford, UK). MTs were mineralized in 2 ml of 2% HNO_3 added with 50 μg/l of indium (used as internal standard). After few minutes of treatment in an ultrasonic bath, a clear solution was obtained which was analysed for the heavy metal content. For quantitative determination, the isotopes of zinc, cadmium, copper, iron and indium were measured at m/z 66, 111, 65, 56, and 115, respectively. Concentration values were corrected respect to indium signal.

2.3. Cloning, expression and purification of MT-A and MT-10

The full-length coding sequences of MT-A and MT-10 genes (GenBank Accession No. AAA49565 and AY566248) were obtained as previously reported [20,21]. Recombinant MT-A from *O. mykiss* and MT-10 from *M.*