Journal of Inorganic Biochemistry 127 (2013) 253–260

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

The major function of a metallothionein from the aquatic fungus *Heliscus lugdunensis* is cadmium detoxification ☆

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ARTICLE INFO

Article history:
Received 6 February 2013
Received in revised form 31 May 2013
Accepted 2 June 2013
Available online 7 June 2013

Keywords:
Aquatic fungus
Metallothionein
Cd(II) detoxification
Electronic absorption spectroscopy
NMR spectroscopy

ABSTRACT

A spring from a former copper shale mine in the area of *Mansfelder Land*, Germany, shows extremely high transition metal ion concentrations, *i.e.* 40 mM Zn^{II}, 208 μM Cu^{II}, 61 μM As^{V}, and 25 μM Cd^{II}. This makes it a challenging habitat for living organisms as they have to cope with metal ion concentrations that by far exceed the values usually observed in spring water. One of the surviving species found is the aquatic fungus *Heliscus lugdunensis* (teleomorph: *Nectria lugdunensis*). Investigation of its redox related heavy metal tolerance revealed the presence of small thiol containing compounds as well as a small metallothionein, Neclu_MT1 (MT1_NECLU: P84865). While Cd^{II}-induction of metallothioneins is observed in many species, the fact that exclusively Cd^{II}, but not Zn^{II}, Cu^{I}, As^{III} or oxidative stress can induce Neclu_MT1 protein synthesis is unparalleled. To complement the physiological studies performed in the fungus *H. lugdunensis*, the Cd^{II} and Zn^{II} binding characteristics of the recombinantly expressed protein were spectroscopically analysed *in vitro* aiming to demonstrate the observed Cd^{II} specificity also on the protein level. Stoichiometric analyses of the recombinant protein in combination with photospectrometric metal ion titrations and ^{113}Cd-NMR experiments reveal that metal ion binding capacities and consequently the structures formed at physiological Neclu_MT1 concentrations differ from each other. Concluding, we describe the first solely Cd^{II}-inducible metallothionein, Neclu_MT1, from *H. lugdunensis*, featuring a difference in the structure of the Cd^{II} *versus* the Zn^{II} metalated protein in a physiologically relevant concentration range.

1. Introduction

The kingdom of fungi includes a puzzling variety of diverse organisms, populating some of the harshest terrestrial and aquatic habitats, including metal contaminated sites like present and former mining areas [1–4]. This raises the question, which mechanisms allow fungi to cope with such high metal ion concentrations. In *Saccharomyces cerevisiae* the tight regulation of essential Cu^{I} ions, which become toxic at higher concentrations, involves two metal sensing transcription factors, ACE1 and MAC1, as well as the Cu^{I}-specific metallothionein (MT) Cup1 [5]. *Schizosaccharomyces pombe* produces the MT Zym1 as well as the Zn^{II} transporter Zhf in response to Zn^{II} [6]. In many organisms non-essential or toxic metal ions including Cd^{II} are handled on a metabolic level *via* phytochelatin synthase (PCS) [7]. PCS uses heavy metal coordinated glutathione, an important thiol containing cellular redox state regulator, as a substrate to form poly(γ-glutamylcysteine) glycine (phytochelatin, PC: general structure $(\gamma\text{-EC})_nG$, n = 2–6). Some fungi like *S. cerevisiae* and *Heliscus lugdunensis* (unpublished data, G.-J. Krauss) lack the enzyme PCS and hence are not capable of PC synthesis *via* PCS. In *H. lugdunensis*, exposure to Cd^{II} leads to an increase of the intracellular concentrations of glutathione and of the dipeptidic glutathione precursor γ-glutamylcysteine (γ-EC) as well as to the production of a phytochelatin (PC2) possibly formed by a carboxypeptidase [2] and of the metallothionein Neclu_MT1 as defense mechanism [2,8].

MTs are relatively small proteins (<10 kDa) with a high Cys content that can reach up to 33% in the mammalian forms and are found in all living phyla [2,9–12], except *archaea*. Their exceptionally high affinity towards the soft d^{10} metal ions, *e.g.* both essential Zn^{II} and Cu^{I} ions and non-essential Cd^{II} and Hg^{II}, and their large metal ion binding capacity distinguish MTs from other metalloproteins. For the divalent metal ions, the binding strength increases in the order $Zn^{II} < Cd^{II} < Hg^{II}$ in line with increasing thiophilicity of the metal ions [13]. Generally, MTs are proposed to play a role in Zn^{II} and Cu^{I} homeostasis as well as heavy metal ion detoxification [14], and it is often not easy to separate and decipher these two functions from each other due to the nearly unprecedented high thiol content of these proteins: whenever an organisms is challenged with high concentrations of *e.g.* Hg^{II} or Cd^{II}

☆ This work is dedicated to the memory of Professor Ivano Bertini.

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http://dx.doi.org/10.1016/j.jinorgbio.2013.06.001

any MT present will take up these thiophilic metal ions like a sponge owing to the high thermodynamic stability of the corresponding Hg^{II}- and Cd^{II}-complexes. However, this "chemical" property can possibly mask a homeostatic function, *i.e.* binding of Zn^{II} or Cu^{I} in the unchallenged organism [13]. Recently, even an Ag^{I} containing MT was isolated from the fruit bodies of an ectomycorrhizal fungus, which was grown on naturally Ag^{I}-contaminated soil [15], further expanding the list of metal ions found associated with MTs *in vivo*. Additional yeast complementation assays showed that this MT can also function in Cu^{I} resistance. Again this leaves the unresolved question, whether this specific MT has physiologically a dual function, including heavy metal detoxification, or was just "incidentally" loaded with Ag^{I} ions due to the higher thermodynamic stability of the resulting Ag^{I}–MT complex. This particular example further illustrates the difficulty of classifying MTs into metal homeostasis or metal detoxification linked proteins. Recently an approach to separate Zn^{II} homeostasis from Cd^{II} detoxification linked MTs was proposed based on the presence of histidine residues in the amino acid sequence as a contributing factor [16,17]. This hypothesis is corroborated for example by the seed-specific MT E_c-1 from *Triticum aestivum* (common bread wheat). This protein was isolated directly from plant material and contains exclusively Zn^{II} ions in its native state [18] suggesting an involvement of E_c-1 in zinc storage for germination. The solution structure of the protein solved by NMR spectroscopy revealed that the two highly conserved His residues participate in Zn^{II} binding [19], and hence a role of these ligands in the discrimination against Cd^{II} was proposed. Moreover, studies of a Zn-finger peptide with a Cys_2His_2 binding site have shown that gradual replacement of His residues by Cys increases the stability of the Cd^{II} complexes, while the binding constants of the Zn^{II} forms remain roughly the same [20].

The focus of the present study is set on the Cd^{II} specificity of the metallothionein Neclu_MT1 from the fungus *H. lugdunensis*, which belongs to the group of aquatic hyphomycetes. Two strains, H 8-2-1 and H 4-2-4, were isolated from a heavily metal ion polluted spring of a former copper mining district in the area of *Mansfelder Land*, Germany [21]. The small MT Neclu_MT1 was first described in the *H. lugdunensis* strain H 8-2-1 as part of the thiol-based defense mechanism of the fungus under the high Cd^{II} concentration of 25 μM observed at the site of isolation. Interestingly, only Cd^{II} was found bound to Neclu_MT1 *in vivo* [8] raising the question if Cd^{II} or other metal ions can also induce the synthesis of Neclu_MT1. While induction by Cd^{II} ions was shown for many *mt* genes from diverse organisms [22–25], results are only scarcely followed through to their logical conclusion: the actual evaluation of MT protein levels in the organism [26,27]. Quantification of protein levels in the host organism is much more complex and tedious experimentally compared to gene expression studies, however, results have the by far greater biological significance.

In order to obtain a deeper insight into the regulation and detoxification mechanisms of organisms living in heavily Cd^{II} polluted environments, we analysed the metal ion binding and discrimination properties of Neclu_MT1 in more detail. In addition, results from complementary experiments testing the Neclu_MT1 levels in *H. lugdunensis* in response to the addition of various metal ions are presented.

2. Material and methods

2.1. Materials

All reagents used in the experimental procedures were of high or highest grade or purity.

2.2. Physiological experiments with H. lugdunensis strain H 4-2-4

Isolation from its native site, *in vitro* growth, and induction conditions as well as quantification of metal ions and cellular thiol compounds using post-column derivatisation RP-HPLC has been described in detail before [4,8,28]. In summary, the fungus was precultivated for five days on malt agar to ensure identical starting conditions for metal ion supplementation experiments. For the intracellular metal ion accumulation experiments *H. lugdunensis* was grown in liquid culture containing 25 μM Cd^{II} or 25 μM Zn^{II}, respectively. Subsequently, mycelia were washed, dried, and digested with acid before quantification of metal ion contents with flame atomic absorption spectroscopy (F-AAS, ATI Unicam). For the evaluation of Neclu_MT1 protein levels, culture media were supplemented with 25–50 μM As^{V}, 50–350 μM Zn^{II}, 25–100 μM Cd^{II}, or up to 208 μM Cu^{II}. For the quantification of thiol compounds and Neclu_MT1 protein levels, growth in presence of 25 μM Cd^{II} was compared to control conditions, *i.e.* no addition of Cd^{II}. For quantification, mycelia were harvested, washed twice with distilled water, dried, frozen, ground in liquid nitrogen, extracted with 1 M NaOH containing 1 mg mL^{-1} $NaBH_4$ (15 min, 20 °C), and centrifuged (11 000 × *g*). The supernatant was acidified to pH 1.5 with HCl and incubated for 15 min on ice. After centrifugation (5 min, 11 000 × *g*) thiol compounds were separated by reversed phase HPLC (RP-18 column, linear gradient (20 min, 2–20% acetonitrile in water, pH 3.0 by trifluoroacetic acid), followed by 5 min of 20% acetonitrile, 1 mL min^{-1}) and identified with standard addition experiments using commercially available or recombinantly expressed compounds. Thiol quantification was performed using online-post-column derivatisation with 0.3 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) in 50 mM KH_2PO_4, pH 8.0, 0.4 mL min^{-1} and detection of modified thiols at 410 nm. Quantification of thiol compounds and determination of metal ion concentrations were performed in at least five replicates at the log phase stage, *i.e.* after 3 days. To determine the total cellular concentrations, the dry weight of mycelia was subtracted from the fresh weight and the difference used as volume for the calculations. In all experiments resazurin (7-hydroxy-3*H*-phenoxazin-3-one 10-oxide) as well as wet- and dry weight was used to assay fungal viability.

2.3. Plasmid construction, MT production, purification, and preparation of apo-Neclu_MT1

For the synthetic gene encoding the Neclu_MT1 protein two 75 nt long oligonucleotides were purchased from metabion GmbH (Martinsried, Germany) (forward: 5′-tctccgtgca cctgctctac ctgcaactgc gcgggtgcgt gcaactcttg ctcttgcacc tcttgctctc acatg-3′; reverse: 5′-gtga gagcaa gaggtgcaag agcaagagtt gcacgcaccc gcgcagttgc aggtagagca ggtgc acgga gacat-3′). BamHI and EcoRI restriction sites were introduced by PCR and the digested product cloned into the pGEX-4T expression vector (GE Healthcare). The GST-Neclu_MT1 fusion protein was overexpressed in *Escherichia coli* BL21(DE3) cells and protein purification was performed as described in [29], *i.e.* after washing, the fusion protein was stripped from the GSH-Sepharose column (GE Healthcare) with 10 mM glutathione. The fusion protein treated this way contained on average 1.7 equiv. Zn^{II} or 2.5 equiv. Cd^{II} ions. After thrombin cleavage a final protein with a length of 25 amino acids and the following sequence

GSPCTCSTCNCAGACNSCSCTSCSH

was obtained.

The N-terminal amino acid Gly is generated by the thrombin cleavage site. Preparation of a completely reduced and demetalated monomeric apo-Neclu_MT1 species was performed according to [30].

2.4. Preparation of Cd^{II}- and Zn^{II}-Neclu_MT1 for mass spectrometry (MS), Chelex® 100, and size exclusion chromatography (SEC) experiments

All solutions used in the protein reconstitution and metal-ion titration experiments were rendered oxygen free by three freeze-pump-thaw cycles on a vacuum line, saturated with argon, and prepared or