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ORIGINAL PAPER

Stress response in tardigrades: differential gene expression of molecular chaperones

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Abstract Semi-terrestrial tardigrades exhibit a remarkable tolerance to desiccation by entering a state called anhydrobiosis. In this state, they show a strong resistance against several kinds of physical extremes. Because of the probable importance of stress proteins during the phases of dehydration and rehydration, the relative abundance of transcripts coding for two α-crystallin heat-shock proteins (Mt-sHsp17.2 and Mt-sHsp19.5), as well for the heat-shock proteins Mt-sHsp10, Mt-Hsp60, Mt-Hsp70 and Mt-Hsp90, were analysed in active and anhydrobiotic tardigrades of the species *Milnesium tardigradum*. They were also analysed in the transitional stage (I) of dehydration, the transitional stage (II) of rehydration and in heat-shocked specimens. A variable pattern of expression was detected, with most candidates being downregulated. Gene transcripts of one *Mt-hsp70* isoform in the transitional stage I and *Mt-hsp90* in the anhydrobiotic stage were significantly upregulated. A high gene expression (778.6-fold) was found for the small α-crystallin heat-shock protein gene *Mt-sHsp17.2* after heat shock. We discuss the limited role of the stress-gene expression in the transitional stages between the active and anhydrobiotic tardigrades and other mechanisms which allow tardigrades to survive desiccation.

Keywords Alpha-crystallin protein · Anhydrobiosis · Cryptobiosis · Heat-shock protein · Tardigrada · *Milnesium tardigradum*

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Introduction

Along with nematodes and rotifers, semi-terrestrial tardigrades exhibit a remarkable tolerance against almost complete desiccation by entering a state known as anhydrobiosis (Keilin 1959) in all developmental stages (Schill and Fritz 2008). To survive drought, which occurs frequently in the habitat of moss-dwelling tardigrades, they enter a so-called tun state (Baumann 1922) and show strong resistance to physical extremes, including high and low temperatures (Ramløv and Westh 1992; Sømme 1996; Ramløv and Westh 2001; Hengherr et al. 2009), high pressure (Seki and Toyoshima 1998), vacuum and ionising irradiation (Horikawa et al. 2006; Jönsson et al. 2008). In the anhydrobiotic state, their metabolism is barely measurable (Pigoń and Węglarska 1955). The longer the animals spend in this state of suspended animation, the longer their lifespan (Hengherr et al. 2008a). The animals resume activity after successful rehydration.

Due to the remarkable ability of tardigrades to survive extreme desiccation, few studies on stress proteins (Schill et al. 2004; Jönsson and Schill 2007) have been carried out to investigate the molecular mechanisms of anhydrobiosis. One isoform of a heat-inducible heat-shock protein (Hsp70) has been described by Schill et al. (2004) as upregulated in the transition phase from the active to the anhydrobiotic state in *Milnesium tardigradum*.

Additionally, in the species *Richtersius coronifer*, a higher protein level of Hsp70 was detected during the transition from the anhydrobiotic to the active state (Jönsson and Schill 2007), whereas a decreased level of Hsp70 was found in anhydrobiotic animals. The investigation of transcripts and encoded stress proteins is based on their well-known function as molecular chaperones (Gething and Sambrook 1992; Georgopoulos and Welch 1993; Jakob et al. 1993). Results derived from several other organisms that tolerate dehydration or suspended animation like nematodes (Chen et al. 2006), crustaceans (Liang et al. 1997b; MacRae 2003), insects (Tammariello et al. 1999; Hayward et al. 2004; Bahrndorff et al. 2008; Lopez-Martinez et al. 2009) and plants (Alamillo et al. 1995; Ingram and Bartels 1996) suggest a versatile role for the stress response in dormant stages.

The present study examines whether an hsp stress response in anhydrobiotic tardigrades operates during dehydration and rehydration. Therefore the expression of several *hsp* transcripts belonging to different Hsp groups was analysed in the eutardigrade *M. tardigradum*. The sequences were taken from our expressed sequence tag (EST) library based on mRNA originating from specimens of *M. tardigradum* from our tardigrade culture. Transcripts of the chaperonin *Mt-shsp10* gene, two α-crystallin small heat-shock protein genes (*Mt-sHsp17.2* and *Mt-sHsp19.5*), one *Mt-hsp60* gene, three *Mt-hsp70* genes, as well as one Mt-*hsp90* gene, were examined to cover a broad range of heat-shock protein genes.

Materials and methods

Tardigrade culture

The study was carried out on the cosmopolitan eutardigrade species *M. tardigradum* Doyère 1849 (Apochela, Milnesidae). Tardigrades were and reared on petri dishes (ø 9.4 cm) filled with a small layer of agarose (3%; peqGOLD Universal Agarose, peqLAB, Erlangen, Germany) and covered with spring water (Volvic™ water, Danone Waters Deutschland, Wiesbaden, Germany) at 21°C and a light/dark cycle of 12 h. Rotifers (*Philodina citrina*) and nematodes (*Panagrellus* sp.) were provided as a food source, and juvenile tardigrades were also fed with the green alga *Chlorogonium elongatum*. For all experiments, adult animals in good physical condition were taken directly from the culture and starved for 3 days to avoid extraction of additional RNA from incompletely digested food in the intestinal system.

Experimental design

To investigate differences in the expression of stress genes during anhydrobiosis, four different groups of tardigrades were set up. Expression of stress transcripts was analysed during the transition from the active to the anhydrobiotic animals (transition stage I), both during the anhydrobiotic stage and during the transition from the anhydrobiotic to the active state (transition stage II). Active animals were used as a control group. An additional group of animals was analysed, in which the animals were exposed to thermal stress for 1 h at 37°C in a heating block (Thermomixer 5436, Eppendorf, Hamburg, Germany). The transition stages were defined as described earlier by Schill et al. (2004) as transitional stage I, in which animals had started tun formation by contracting their legs, and transitional stage II, in which animals showed distinct movements and had stretched their legs. To achieve desiccation, they were put in open microlitre tubes (Sarstedt, Nümbrecht, Germany) and into small plastic chambers with 85% relative humidity (RH) for 2 days. Subsequently, the tubes were transferred for seven days into chambers with 33% RH to desiccate the animals. The humidity levels described above were achieved by sustaining a constant saturation vapour pressure over a saturated salt solution of KCl and $MgCl_2$, respectively. The boxes had transparent tops to monitor the processes without changing the humidities.

RNA preparation and quantitative real-time PCR

Each experimental group described above consisted of 50 animals, which were subdivided into groups of ten animals. Before RNA isolation, the animals of the "active" and "transition I" group were washed three times in spring water (Volvic water™, Danone). "Anhydrobiotic" and "transition II" animals were washed before desiccation. The tardigrades were homogenised in lysis buffer using a bead mill (FastPrep 24, MP Biomedicals, Heidelberg, Germany). Total RNA was prepared with the RNeasy® Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was digested during the preparation with the included DNAse I. The RNA was eluted in RNAse-free water, and quantity and quality were checked with a NanoDrop® ND-1000 spectrophotometer (peqLab, Erlangen). Subsequently, cDNA was prepared with the cDNA Synthesis Kit from Bioline (Luckenwalde, Germany).

To measure relative expression of the transcripts of the experimental groups compared to the control group,

quantitative real-time PCR was performed with a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, München, Germany), and 0.5 µL of the first strand cDNA synthesis reaction mixture was used as template in a total reaction mix of 25 µL (ImmoMix™, Bioline) with 2.0 mM $MgCl_2$. Due to the fact that ribosomal proteins represent adequate housekeeping genes in quantitative real-time PCR (qrtPCR) in general (de Jonge et al. 2007), a partial sequence of the ribosomal protein S13 gene (*rps13*) was used as reference gene in this study. The efficiency of the PCR reactions was calculated from the slope of the standard curve, which was derived from a dilution series (1:2, 1:20, 1:200 and 1:2,000). Every PCR reaction was performed in triplicate. Threshold cycles (C_t values) were calculated by the MyiQ 2.0 software and analysed with the freely available Relative Expression Software Tool (REST©; Pfaffl et al. 2002), which allows for a determination of significant differences between the expression ratios and the estimation of the standard errors. The $\log_2$ expression ratios of the experimental groups were plotted to compare with the control group; error bars represent the $\log_2$ values of the standard error. Significantly different expression between experimental groups and control groups was accepted for $P<0.05$ (Pair Wise Fixed Reallocation Randomisation Test©, implemented in REST©).

The following programme was routinely used to conduct the qrtPCRs: initial denaturation step (95°C for 10 min) followed by 40 cycles of denaturation (10 s), annealing (20 s) and elongation (20 s). A melt curve analysis was added (95°C to 55°C in steps of 0.5°C every 30 s), and the product size was subsequently examined by gel electrophoresis.

Primers were designed by using the free internet tools "Primer3" (Rozen and Skaletsky 2000) and "NCBI/Primer BLAST" (based on Primer3) with target sequences from *M. tardigradum* EST libraries. Computational sequence analysis of the deduced EST sequences was performed using the Basic Local Alignment Search Tool (Altschul et al. 1990) at the web pages of the National Center for Biotechnical information (NCBI). Sequences with the highest homology were aligned with ClustalW implemented in the software MEGA4 (Tamura et al. 2007), and ESTs were named after GenBank entries with the highest homologies: *Mt-shsp10*, *Mt-Hsp17.2*, *Mt-Hsp19.5*, *Mt-hsp60* and *Mt-hsp90*. Three different ESTs resulted in significant alignments with proteins of the Hsp70 family and were named *Mt-hsp70-1*, *Mt-hsp70-2* and *Mt-hsp70-3*. Primers were designed for the coding sequences, without considering 3′ and 5′ untranslated regions.

Results

Analyses of two stress-gene sequences in our EST library resulted in the complete open reading frames for putative proteins with a high homology to small heat-shock/α-crystallin proteins. Alignment of the two sequences (Fig. 1), termed *Mt-shsp*17.2 and *Mt-shsp*19.5 after their calculated size of 17.2 and 19.5 kDa, respectively, display considerable differences between the two sequences and similarities to other α-crystallin/sHsp proteins (Fig. 1). Heat-shock treatment resulted in a 778.6-fold higher transcription level of *Mt-shsp17.2* in animals compared to the control group (Fig. 2), indicating that it codes for an inducible α-crystallin/sHsp protein. In contrast, *Mt-shsp*19.5 was not regulated during heat shock. Both α-crystallin/sHsp sequences were not significantly regulated during the process of anhydrobiosis, with one exception; *Mt-shsp*17.2 was downregulated in the transitional stage II.

In general, most of the genes under investigation were downregulated or regulated at all in the two transitional stages and the anhydrobiotic stage.

Significant down regulation was found for *Mt-hsp60* ($P=0.031$) and *Mt-hsp70-1* ($P=0.025$) in the transitional stage I, whereas *Mt-hsp70-2* ($P=0.019$) was upregulated. In the anhydrobiotic stage, the gene *Mt-hsp90* ($P=0.002$) was significantly upregulated, and all other stress genes showed no significant regulation. However, in the transitional stage II, *Mt-shsp10* ($P=0.002$), *Mt-hsp60* ($P=0.009$), *Mt-shsp19.5* ($P<0.001$), *Mt-hsp70-1* ($P=0.002$) and *Mt-hsp70-2* ($P<0.001$) were significantly downregulated. All genes, except the upregulated *Mt-shsp17.2* ($P=0.008$), showed no significant heat-inducible stress response in heat-shocked animals.

The two small heat-shock/α-crystallin protein sequences, *Mt-shsp17.2* (with a strong induction of expression under heat shock) and *Mt-shsp 19.5* (longer isoform with no induction), were analysed bioinformatically to obtain further insights into their function and difference in induction (Fig. 3). The domain analysis (Fig. 3a, b) shows that both proteins contain an alpha-crystallin domain and have a dimer interface. The sHsps are generally active as large oligomers consisting of multiple subunits and are believed to be ATP-independent chaperones that prevent aggregation and are important in refolding in combination with other Hsps. The potential for multimerization is confirmed for these two sequences by corresponding motifs. However, the longer form leads to a different protein and is no longer in the COG0071/IbpA gene family. Furthermore, the N-terminus (first 60 amino acids) of *Mt-shsp 19.5* is tardigrade specific and has no relatives in other organisms. Prosite motifs support the Hsp signature for both proteins and include only often occurring modification motifs. The longer sHsp protein has several potential phosphorylation modification sites predicted in the N-terminus.

To obtain more insight into the differential behaviour of both proteins, potential interaction partners were predicted using the interaction database STRING (von Mering et al.

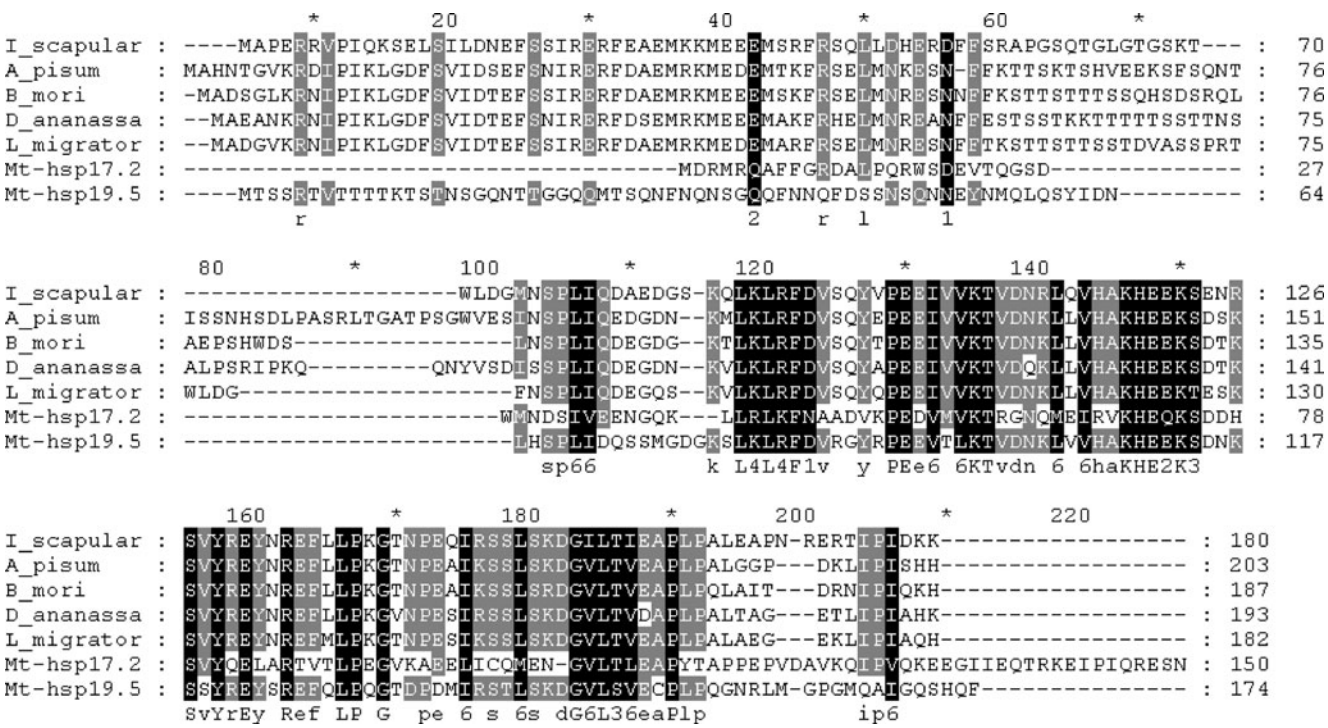

Fig. 1 Alignment of two α-crystallin/small heat-shock proteins from *M. tardigradum* with α-crystallin/small heat-shock proteins from *Ixodes scapularis* (EEC06453), *Acyrthosiphon pisum* (XP_001949446), *Bombyx mori* (NP_001036985), *Drosophila ananassae* (XP_001963454) and *Locusta migratoria* (ABC84493). *Black* high homology, *grey* weak homology, *blank* no homology

2005). Using the hsp homolog for *Mt-shsp 17.2* known from *Drosophila melanogaster* (protein CG14207-PB, isoform B), it appears that there is a tight interaction network in which the *Mt-shsp 17.2* homolog is involved. The protein Mef2 (Myocyte enhancing factor 2) is critical for the regulation of this network and one of the proteins regulated by it is glyceraldehyde 3-phosphate dehydrogenase.

In addition to their developmental function, a number of Mef2 target genes are involved in muscle energy production or storage and were identified in *Drosophila*. As it would be interesting to identify a similar adaptation in tardigrades, we searched by iterative sequence alignment techniques for tardigrade homologues of both proteins. Interestingly, we found the regulatory protein in the

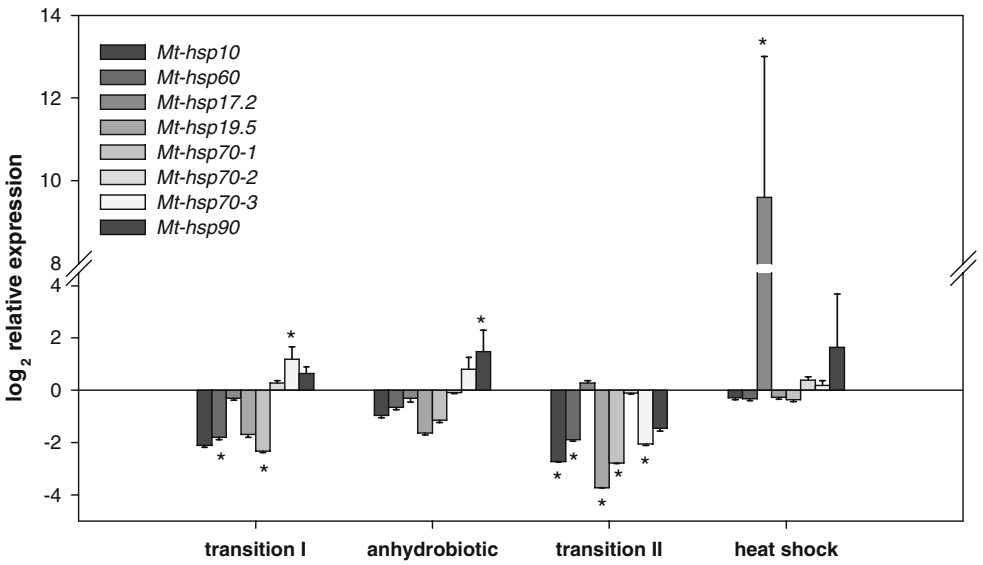

Fig. 2 Relative expression of analysed stress-gene transcripts of *M. tardigradum* in different stages of anhydrobiosis and heat shock. The *asterisks* ($P \leq 0.05$) indicate different expression of transcripts compared with control specimens, which underwent no treatment (displayed by the base line)

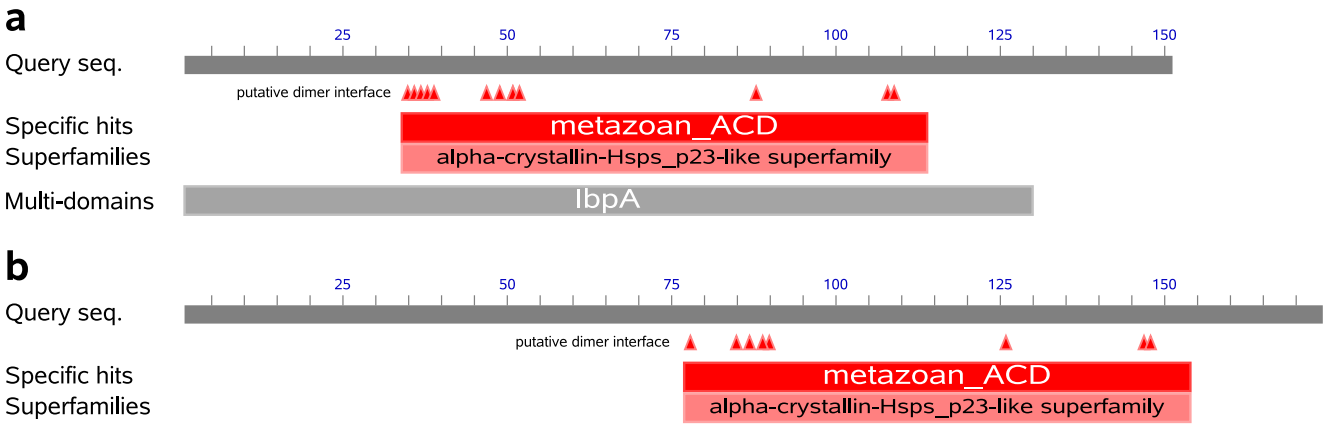

Fig. 3 **a** Domain analysis of *Mt-shsp 17.2* shows that it contains an alpha-crystallin domain (residues 34–113) from the Hsps-p23-like superfamily. There is a putative dimer interface predicted, and residues 1 to 127 belong to COG0071/IbpA, molecular chaperon COG. Compared to other known metazoan proteins, this is a small single domain protein (most others have multidomain context). The closest neighbour by sequence comparison is the heat-shock protein 20.6 (putative) from *I. scapularis* (*e*-value 4e−13) but there are also the well-characterised ones, e.g. from *B. mori* similar over most of the sequence (13–131) with an *e*-value of 2e−12. **b** Domain analysis of *Mt-shsp 19.5* Domain analysis shows that also this protein contains an alpha-crystallin domain (residues 76–154) from the Hsps-p23-like superfamily. At the N-terminal end of the domain, there is again a dimer interface predicted but somewhat weaker. There is highest similarity (1e−33 to heat-shock protein 20.6 isoform 2 from *Nasonia vitripennis*; residues 42–173) but again also to the *B. mori* version (residues 60–156) with 7e−33. Compared to other known metazoan proteins, this is again a small domain protein (most others have multidomain context). However, compared to the shorter version *Mt-shsp 17.2*, we have here a tardigrade-specific N-terminus (first 75 residues) not occurring in other organisms

eutardigrade species *Hypsibius dujardini*. Furthermore, a putative regulatory protein, which could be involved in the network in *M. tardigradum*, has a predicted dual specificity kinase function. Glyceraldehyde 3-phosphate dehydrogenase is found in *M. tardigradum*. In contrast, it turns out that the longer form *Mt-shsp19.5* is not predicted to be involved in this adaptive network. There are no interactions predicted by the STRING database, and furthermore, this is in accordance with our experimental observation that no induction in expression is observed.

Both small heat-shock protein genes were also investigated for regulatory motifs. They contain a number of insignificant motifs in the corresponding untranslated regions. Such patterns with a high probability of occurrence (and which have a high chance of false-positive predictions) include SeCys insertion sequences and GAIT (gamma interferon activated inhibitor of coeruloplasmin mRNA) elements. However, it cannot be ruled out that some type of similar regulation occurs in both of them. Furthermore, the long *shsp* mRNA contains an iron-responsive element structure at position 1188 (see Electronic supplementary material). Here, the chance of occurrence is sufficiently low to suggest functional significance. However, as nothing is known about iron-responsive element-binding proteins in tardigrades and the structure may also be targeted by other proteins, this merely suggests a stability prolonging regulatory element in this region, compatible with the stable, unchanging level of this heat-shock protein mRNA.

Discussion

In this study, the stress response of the eutardigrade *M. tardigradum* was analysed during anhydrobiosis by investigating the expression changes of stress-gene coding sequences for different classes of heat-shock proteins. Sequences were found with significant homologies to several proteins of stress response in EST libraries for *M. tardigradum*. Among them are complete coding sequences for a chaperonin Hsp10 and two α-crystallin/small heat-shock proteins of 17.2 kDa (150 amino acids) and 19.5 kDa (174 amino acids).

Small Hsps prevent protein aggregation and act as molecular chaperones during several kinds of stress (Haslbeck 2002). Studies on sHsp regulation in dormancies of different organisms revealed heterogenous patterns (Bonato et al. 1987; Yocum et al. 1991; Denlinger et al. 1992; Liang et al. 1997a; Yocum et al. 1998; Tammariello et al. 1999; Cherkasova et al. 2000; Goto and Kimura 2004; Rinehart et al. 2007; Gkouvitsas et al. 2008), indicating a diverse array of functions. An essential upregulation of *shsp* has been suggested for cold hardiness in the flesh fly *Sarcophaga crassipalpis* (Rinehart et al. 2007). One of the two *M. tardigradum shsp* sequences, *Mt-shsp17.2*, is strongly inducible by heat-shock treatment, but not regulated during anhydrobiosis. On the contrary, *Mt-shsp19.5* is not inducible by heat and is downregulated in animals in the transition from the anhydrobiotic to the

active state. This leads to the assumptions that both shsps feature different functions. Due to the low expression changes, their role in the anhydrobiosis of tardigrades is questionable, although it is not yet known if there is a sufficient basal level of sHsp proteins in *M. tardigradum*, so that upregulation is not necessary. However, the importance of small heat-shock proteins is clearly demonstrated in *Artemia franciscana*. A massive accumulation of the sHsp p26 occurs in diapausing embryos of this brine shrimp (Liang et al. 1997a; Liang et al. 1997b). The protein p26 is able to move into the nucleus (Clegg et al. 1995) and is thought to protect and/or chaperone, in cooperation with Hsp70, the nuclear matrix proteins (Willsie and Clegg 2002).

This study provides additional data towards the understanding of hsp70 expression during the anhydrobiosis of tardigrades. Schill et al. (2004) described three isoforms of inducible *hsp70* from *M. tardigradum*. The isoform 1 and the isoform 3 did not have a specific function for cryptobiosis. By contrast, transcription of isoform 2 was significantly induced in the transitional stage II between the anhydrobiotic and active stage in *M. tardigradum*. Assuming that a higher mRNA amount may lead to a higher protein content, a functional role of Hsp70 during anhydrobiosis can be suggested, either during anhydrobiosis or as part of a general stress-response mechanism. Since that assumption might not hold, an alternative role might be to prevent protein unfolding and aggregation resulting from the loss of cellular water that takes place during the entry to anhydrobiosis, or in the establishment of a system with refolding capacity to provide functional proteins during and after rehydration.

The lower expression of *Mt-hsp70-1* and *Mt-hsp70-2* during the transition to the active state supports the hypothesis that preceding the actual anhydrobiotic state there is preparation for the time of rehydration. In the eutardigrade species *R. coronifer*, a lower level of Hsp70 protein was found in desiccated animals when compared with active ones (Jönsson and Schill 2007). Assuming that *M. tardigradum* and *R. coronifer* share the same characteristics during desiccation, the upregulated *Mt-hsp70-3* transcript belongs to Hsp70 proteins, which contribute only a small part of the Hsp70 contingent in the cell. Because the antibody used by Jönsson and Schill (2007) was broadly reactive to a wide range of Hsp70 family members, a more prominent Hsp70 isoform might have a higher impact on the overall protein content. However, we note that the low expression of *hsp70* genes and low levels of proteins in tardigrades are similar to data derived from dehydration experiments with yeast containing different amounts of Hsp70 (Guzhova et al. 2008).

Research on Hsp90 revealed many different functions in cells. It acts as a controller of critical hubs in homoeostatic signal transduction, as a regulator of chromatin structure, gene expression, development and morphological evolution and is also involved in the secretory pathway (McClellan et al. 2007; Pearl et al. 2008). Focusing on the role of Hsp90 as a molecular chaperone (Richter and Buchner 2001), the expression changes of a partial putative *Mt-hsp90* sequence were analysed. *Mt-hsp90* was the only sequence investigated in our study that was more abundant in the anhydrobiotic state. However, an increase in its expression was not detected in transitional stage I. In the anhydrobiotic stage, no translation took place, due to the reduced metabolic activity (Pigoń and Węglarska 1955), but a significantly higher level of mRNA was observed, which subsequently decreased after rehydration. If or to what extent the *Mt-hsp90* mRNA was stored for translation into protein during and after rehydration is not known, nor do we know the level required to be effective.

During the whole process of anhydrobiosis, no increased expression was detected for transcripts with high homology to *hsp10* and *hsp60* sequences. Additionally, neither was induced by heat shock at 37°C. Hence, these stress genes, whose proteins are capable of binding and folding non-native proteins (Horwich et al. 2007), which may occur during desiccation, seem to play no relevant role in anhydrobiosis in *M. tardigradum*.

Our investigation of the stress-gene responses in *M. tardigradum* at the transcriptional level clearly shows that most mRNAs are less abundant during anhydrobiosis than in active animals, which may lead to a lower protein level. However, as already mentioned, the levels of stress protein needed for protection or repair in the tardigrade *M. tardigradum* are not known. Focusing on the expression of stress genes, our study suggests a minor role for stabilising and refolding stress proteins, leading to the assumption that denaturation of proteins due to drastic changes during desiccation is not a significant problem for *M. tardigradum*.

The question then arises as to what confers desiccation tolerance on *M. tardigradum* since trehalose (Hengherr et al. 2008b), and stress proteins do not seem to be directly involved. Recent studies showed the existence of other carbohydrates, for example sucrose, sorbitol, inositol and glycerol, have been found in *M. tardigradum* (unpublished results). Those molecules are able to form biological glasses, which may protect cellular structures according to the vitrification hypothesis (Crowe 2002; Crowe et al. 1998). Another important factor might be the presence of late-embryogenesis abundant proteins, which have been detected in *M. tardigradum* (Schill et al. 2004, 2005; McGee et al. 2005; Schokraie et al., submitted) and which are present in many organisms that survive desiccation (e.g. Wise and Tunnacliffe 2004; Goyal et al. 2005; Chakrabortee et al. 2007). A combination of proteins and carbohydrates may also play an important cellular protection role during desiccation in tardigrades.

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