STRESS RESPONSE IN FISH: IDENTIFICATION AND ANALYSIS OF THE EXPRESSION PATTERN OF *hsp70* AND METALLOTHIONEIN GENES IN COMMON CARP (*Cyprinus carpio*)

Ph.D. thesis

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1. Introduction

A sudden temperature upshift and other types of environmental stress induce the synthesis of a specific set of proteins, called heat shock or stress proteins (Hsps). This response, highly conserved throughout evolution, is found universally from bacteria through lower eukaryotes to human. The Hsps themselves and their genes, among the best conserved phylogenetically, comprise several classes; one of them is the Hsp70 family, containing highly conserved and widely studied proteins with a molecular mass of about 70 kDa. Hsp70s play essential roles in the protein metabolism under normal and stress conditions, e.g. *de novo* protein folding, membrane translocation, the formation and disassembly of protein complexes or the degradation of misfolded proteins. They consist of two domains: the 44 kDa N-terminal domain binds and hydrolyses ATP, while the more variable 30 kD C-terminal interacts with unfolded polypeptides. Hsp70s also interact with a number of other proteins, promoting specific chaperoning functions. Their expression is regulated by environmental and physiological stresses and non-stressful conditions such as cell growth and development. Some family members are at best weakly expressed under normal conditions and are inducible by heat and other stresses, allowing cells to cope with acute stressor insults (*bona fide* Hsp70s). Others (Hsp70 cognates, Hsc70s), expressed constitutively, are at best only slightly inducible, and play essential roles in the protein metabolism under normal conditions.

The highly related Hsp70 and Hsc70 are often suggested to have similar physiological functions. Mammals appear to contain more than one isoform for both Hsp70 and Hsc70. There is a higher similarity between the members of the two subfamilies from different species than between Hsp70s and Hsc70s from the same species, e.g. the *hsc70* gene products from human, rat and hamster are 99% similar, while the human Hsp70 and Hsc70 amino acid sequences share only 85% identity. In fish, *Danio rerio* is the only example of variation in the isoforms of Hsc70: the two proteins share 94% identity.

As a consequence of the chemical loading of the environment, living organisms may be exposed to numerous compounds during their lifetime. Cadmium, for example, is now generating concern, due to its accumulation in the environment as a result of industrial practices. Soluble Cd salts accumulate and result in toxicity, causing a variety of adverse health effects, among which kidney dysfunctions, lung diseases and a disturbed calcium metabolism are the most prominent. In an effort to afford protection against the induced

damage, cells respond to toxic metals via activation of the expression of stress genes, other than *hsp*s, e.g. metallothioneins (MTs).

The MTs are a group of low-molecular weight (6-7 kDa), cysteine-rich (30%), inducible intracellular proteins with high affinity for both essential (Zn and Cu) and non-essential (Cd, Pb, As and Hg) metals. The protein-bound metals are situated in two distinct clusters with tetrathiolate coordination. Four decades after the discovery of the MTs as Cd-binding proteins, their functional significance remains unclear. It has been suggested that they are involved in the detoxification of certain heavy metals, the homeostasis of essential trace element ions such as Zn^{2+} and Cu^{2+}, the scavenging of free radicals, and protection against alkylating agents. Consistent with these roles, MT genes in higher eukaryotic species are transcriptionally induced by a variety of stresses, including metals, glucocorticoid hormones, oxidative agents, cold exposure and irradiation.

The distribution of the MTs in virtually all types of organisms studied to date attests to the conserved nature of the MTs and their function. The amino acid sequences of various species exhibit regions of high similarity; conserved nucleotide sequences exist for both the coding region and the regulatory elements of the MTs of mammals, fish and invertebrates.

As compared with studies of stress proteins in bacteria, yeasts and mammals, studies on fish are still in the descriptive stages of documenting novel proteins produced in various tissues in response to a variety of stressors. A majority of the data were obtained from *in vitro* studies which concentrated on the protein expression pattern. In the first part of the present study, we summarize our results relating to the identification of two *hsc70* and one *hsp70* genes isolated from the common carp (*Cyprinus carpio*) and studies of the expression patterns of these genes in a variety of tissues under different stress conditions. In the second part of the study, we focus on the function of *hsp70*s and metallothionein in the protection of the heart against temperature and chemical stresses. All experiments were carried out *in vivo,* on adult animals.

2. Materials and methods

Animals and treatment

Carp (*Cyprinus carpio*) weighing 500-1000 g, obtained from the Tisza Fish Farm, Szeged, were acclimatized under fasting conditions in well-aerated 400-l water tanks, over a 2-3-week period at 12-15 ^{o}C. For treatment with Cd, Cu and As, the carp were transferred into 100-l

water tanks containing 1 or 10 mg/l of metal ion under static conditions. In heat shock treatment, the fish were exposed to a temperature 14 ^{O}C higher than the acclimatization temperature, and in cold shock experiments to 5 ^{O}C, for 1 to 5 h. Samples were taken from the liver, kidney and brain, either immediately after the various heat or cold treatments, or after a 1-h recovery period at the acclimatization temperature. In all experiments, 3 or 4 animals were sacrificed for organ harvesting at each time point. Tissues were removed, frozen immediately in liquid nitrogen, and stored at –80 ^{O}C.

Standard molecular biology techniques

- Preparation of genomic and plasmid DNA
- Digestion with restriction enzymes and agarose gel-electrophoresis
- Cloning into plasmid vectors and transformation
- RNA extraction, reverse transcription and PCR amplification
- Radioactive DNA labelling and Northern hybridization
- Sequencing and sequence analysis

3. Results and discussion

The genes of three 70 kDa stress proteins were identified in the carp. Beyond the high sequence homology, typical for stress proteins, isolation of the genes was based on literature data indicating that the *hsp70* structure genes isolated from vertebrates so far have no intron sequences; and that *hsp70* mRNA can not, or only in minimal amount, be detected in a stress-free state. The genes were classified as constitutive (*hsc70*) or inducible (*hsp70*) by sequence comparison, phylogenetic analysis, gene structure and expression pattern. Accordingly, two of the genes code Hsc70 and one codes Hsp70 proteins.

In mammals, the presence of several *hsc70* genes in a species is common. In the fish, however, the carp *hsc70* genes are merely the second example of this phenomenon, and the first example of substantial sequence differences within the lower vertebrates (88% homology, in contrast with 96-98% in other species).

The structural genes *hsc70-1* and *hsc70-2* have an exon/intron structure, whereas *hsp70* has no introns. The identified genome region of *hsc70-1* has 8 exons and 7 introns, with evolutionarily conserved exon/intron boundaries.

The expression of the three genes was investigated in various organs (liver, kidney, brain and muscle) of untreated fish. The typically tissue-specific expression of the carp *hsc70*

genes is a novelty: in some tissues both forms of mRNA are detected, but in others one or the other is expressed in a complementary pattern.

The expression of *hsc70-1* was maximal in the muscle, where the amount of its mRNA was comparable with that of β-actin, whereas the amount of *hsc70-2* mRNA was at 15% of that of β-actin. In the liver and kidney, the *hsc70-1* gene products were at or below the detection limit. The expression of *hsc70-2* was maximal in these organs; the amount of mRNA was 55-60% of that of β-actin. In the brain and the heart, the two gene products were present in approximately equal amounts. *hsp70* was not detected in the brain. In the kidney, liver and muscle, its level was around the detection limit, with strong individual variations.

The inducibility of the three genes by temperature alteration was investigated. A sudden increase of the water temperature by 14 or even 18 °C resulted in moderate increases in the expressions of the three genes. An important difference between the *hsp70* and *hsc70* genes is that constitutively expressed genes are not directly induced by heat shock. Only after the recovery time was an increase measured in the mRNA. The expression of the *hsp70* gene, on the other hand, was the same on direct heat shock and after recovery. The 4 to 4.5-fold induction of *hsc70-2* is considerable as compared to the heat stress response of the constitutive *hsc70* genes of other species.

The effects of cold shock on the expressions of the *hsp70/hsc70* genes were also studied. It is reasonable to suppose that the organism needs protection against sudden temperature drops which necessitates the increased synthesis of *hsc/hsp70*. A cold effect itself can denature proteins, and *hsp/hsc70* products are essential in “refolding”. Moreover, the metabolic processes slowed by the cold shock are accelerated during recovery, and new proteins are synthesized, where *hsp70/hsc70* are again required. Lowering the water temperature induced clear, tissue- and gene-specific alterations. In the muscle, the expression of *hsc70-1* was largely inhibited, but *hsc70-2* and *hsp70* were induced. The 7.5- to 10-fold increase in the *hsc70-2* mRNA level is virtually unique: such an induction of the *hsc70* expression has been measured so far only in NIH3/T3 and human HeLa cell lines treated with azetidine (an amino acid analogue). The substantial decrease in the expression of the muscle-specific *hsc70-1* and the strong induction of *hsc70-2* (barely detectable in the normal state) suggest some kind of task specialization of the two genes in both the untreated and stressed

state. The distribution among the *hsc70* genes of a species has been described in the literature, but such a stress-dependent phenotype seems to be a novum.

Besides the temperature changes, the effects of cadmium, an agent causing general protein damage, were investigated by using two different modes of metal administration. When Cd was applied in the water at a concentration of 10 mg/l, the expression of *hsp70* was not or only moderately influenced. The fact that an increase (2.5-fold maximum) in the *hsp70* mRNA level was seen in the liver and muscle only after a 96-h exposure may be due to the efficient induction of MT gene expression in nearly all tissues by this concentration of Cd. The synthesis of MTs (proteins of high metal-binding capacity), however, may need an increased activity of the "foldosomes," which would then explain the increase of *hsc70* expression in the liver, the centre of detoxification (11- to 13-fold for *hsc70-1*).

When Cd was administered intraperitoneally in dose of 10 mg/kg, the reaction was completely different. The expression of *hsp70* gene rose dramatically (15-fold 10 h after treatment), but the *hsc70* genes were much less expressed than in the case of 10 mg/l Cd in the water. The MTs do not provide protection against such high doses of Cd, and an increased amount of proteins will be denatured, with possible substantial damage to the transcriptional/translational apparatus.

The expressions of the "stress" genes *hsp70, hsc70-1*, MT-1 and MT-2 were investigated in one organ, the heart, after temperature and metal stress. A temperature increase of 14 °C increased the expression of the *hsp70* gene 5-fold. After the recovery period, a 10-fold increase in the level of *hsp70* mRNA was seen. The expression of *hsc70-1* was also increased (3-fold maximum) by the temperature rise. Thus, the induction of the *hsp70* gene by heat shock was much stronger than in the liver or the striated muscle. The expressions of the MT genes were not influenced by temperature change.

The effects on the expression of the "stress" genes of three heavy metals were also investigated: Cd - highly toxic, As - toxic, and Cu - essential in small amounts, but toxic in higher amounts. Given in a dose of 10 mg/l, Cd induced a low, but long-lasting increase in the expression of *hsp70*. The level of *hsc70-1* mRNA was not altered, just as in the striated muscle. As and Cu in the same dose induced both genes in different ways from each other and from the action of Cd. As induced a temporary, mild induction of *hsp70,* as opposed to the low but protracted effect of Cd, and the continuous induction by Cu lasting up to the end of the experimental period. The extents of *hsp70* induction by the three metals followed the

sequence Cu>Cd>As.The *hsc70-1* gene was not induced by Cd, whereas it was induced 2-fold by As, and 4-fold by Cu. The results suggest that, of the two proteins, the constitutive form is of greater importance in the defence against the harmful effects of As and Cu.

In the heart, all three metals induced the expressions of the two MT genes examined. The continuous presence of Cd (10 mg/l) caused a 1.7- to 2.5-fold induction of MT-1, while a single dose of 10 mg/kg led to an 8- to 12-fold induction. In the event of continuous exposure, the induction of MT-2 was stronger (5-fold). Continuous treatment of the carp with As and Cu induced the similarly high (10- to 15-fold maximum) induction of MT-1. The induction of MT-2 by Cu was only half of that by As.

To summarize the above results, it can be stated that the stress response evoked by heavy metals in the heart is a complex phenomenon. In both gene families, the stress response was dependent on the metal and the mode of exposure. The increased synthesis of MT proteins is important in the protection of the proteins constituting the cell, as evidenced by the relatively low induction of the heat shock genes on heavy metal treatment. The increase in the mRNA of the constitutively expressed *hsc70-1* possibly indicates a higher foldosome activity. It is interesting that the induction of *hsp70* and *hsc70-1*, indicating the denatured state of the proteins, is higher with the essential metal Cu than with the highly toxic metals As and Cd. A possible explanation of this can be derived from the functions of the MT proteins, binding and distributing the essential metals Zn and Cu, and thereby maintaining the metal homeostasis of the cells. The literature data demonstrate that oxidative stress of the heart results in the intracellular release of Zn ions from the MT proteins, leading to a several fold increase in the intracellular free metal concentration. Cu ions, like Zn ions, are bound with low affinity by MTs, and the free metals can cause protein denaturation and stress protein induction.

list of Publication

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