The Journal of Experimental Biology 200, 607–614 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JEB0525

SYNTHESIS OF STRESS PROTEIN 70 (Hsp70) IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) RED BLOOD CELLS

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Accepted 1 November 1996

Summary

Unlike enucleated mammalian red blood cells (rbcs), the nucleated rbcs of lower vertebrates are capable of protein synthesis and may, therefore, serve as a valuable model to investigate the adaptive significance of stress protein synthesis in cells. This study examined the synthesis of stress protein 70 (Hsp70) in rbcs of the temperature-sensitive rainbow trout *Oncorhynchus mykiss* in response to heat shock and anoxia. Through western blot analysis, we have demonstrated that rainbow trout rbcs synthesize Hsp70 both constitutively and in response to an increase in temperature. Radioisotopic labelling experiments indicated that the temperature at which Hsp70 synthesis was induced in fish acclimated to 10 °C was between 20 and 25 °C. Actinomycin D blocked *de novo* Hsp70 synthesis, implying that synthesis of Hsp70 is regulated at the level of transcription in rainbow trout rbcs. Since trout rbcs rely heavily on aerobic metabolism, but may also experience very low oxygen levels within the circulation, we also examined the relative importance of (1) anoxia as a stimulus for Hsp70 synthesis and (2) oxygen as a requirement for protein synthesis under control and heat-shock conditions. We found that trout rbcs were capable of protein synthesis during 2 h of anoxia, but did not increase Hsp70 synthesis. Moreover, rbcs subjected to combined anoxia and heat shock exhibited increases in Hsp70 synthesis that were similar in magnitude to those in cells exposed to heat shock alone. The latter results suggest that rainbow trout rbcs are (1) able to synthesize non-stress proteins during anoxia, (2) capable of tolerating periods of reduced oxygen availability without increased synthesis of stress proteins and (3) able to maintain the integrity of their heat-shock response even during periods of anoxia.

Key words: *Oncorhynchus mykiss*, rainbow trout, stress proteins, Hsp70, temperature, oxygen, anoxia, actinomycin D, transcription.

Introduction

Environmental stresses, such as heat shock, toxic metal contamination and hypoxia/anoxia, may be commonly experienced by many aquatic poikilothermic organisms. In order to minimize the potentially detrimental effects of these stresses, organisms are capable of synthesizing a group of proteins known as stress proteins or heat-shock proteins. Generally, these proteins are thought to provide the cell with protection by preventing aggregation or improper folding of proteins (see Gething and Sambrook, 1992; Parsell and Lindquist, 1993; Becker and Craig, 1994; Wynn *et al.* 1994; Hartl, 1996), thereby ensuring the survival of the organism under stressful conditions by suppressing cell damage and/or cell death (Morimoto *et al.* 1995*a*). In addition to being induced by stress, these proteins have essential functions under normal growth conditions and are classified as part of a larger family of molecular chaperones (Hartl, 1996). Thus, stress proteins, or molecular chaperones, prevent the formation of misfolded protein structures, both under normal conditions and when cells are exposed to stress.

The nucleated rbcs of fish possess several unique characteristics making them an ideal system to study the adaptive significance of stress protein synthesis. First, unlike their enucleated mammalian counterparts, fish rbcs have high levels of aerobic metabolism (Boutilier and Ferguson, 1989) which supports many cellular processes, including protein synthesis (Speckner *et al.* 1989). Second, blood is easily collected and experimentally manipulated, and many cellular functions and experimental approaches have been well-described. Third, blood could serve as a valuable, non-terminal means of assessing fish stress in natural or aquaculture environments. Finally, the protective role of stress proteins within the rbcs of an active, highly temperature-sensitive species, such as the rainbow trout, may be particularly critical to preserve rbc function under stressful conditions and to ensure adequate delivery of oxygen to the tissues.

Hypoxia and anoxia have been shown to elicit the stress response in several tissues (Hammond *et al.* 1982; Kobayashi and Welsh, 1995; Mestril *et al.* 1994; Myrmel *et al.* 1994; Sciandra *et al.* 1984; Tuijl *et al.* 1991). Unlike the cells of many tissues, however, the rbcs of an active species such as the

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rainbow trout may frequently be exposed to brief periods of hypoxia and/or anoxia during their transit through the capillaries of some tissues (e.g. white muscle) and through the venous circulation, particularly following episodes of intense activity. Thus, the importance of oxygen deprivation for stress protein synthesis in fish rbcs may be quite different from that in most other tissues. Oxygen is an important requirement for the energetically expensive process of protein synthesis in many cell types, including nucleated rbcs. According to Ferguson *et al.* (1989), salmonid rbcs obtain over 90 % of their metabolic energy from aerobic metabolism. To date, however, the importance of oxygen in the ability of rbcs to synthesize stress proteins has not been investigated.

The purpose of this study was to examine the synthesis of Hsp70 in the rbcs of the rainbow trout in response to heat shock and anoxia. Since many aspects of stress protein synthesis have yet to be adequately described in nucleated rbcs, and because rainbow trout are a highly temperature-sensitive species, we first examined the synthesis of stress proteins in rainbow trout rbcs following an acute temperature stress *in vitro*. We then investigated (1) the role of anoxia as a stimulus and (2) the importance of oxygen as a requirement for stress protein synthesis in trout rbcs.

Materials and methods

Animals and surgery

Freshwater rainbow trout, *Oncorhynchus mykiss* (Walbaum) (1–2 kg; *N*=24), were obtained from a commercial fish hatchery (Pure Springs Trout Farm, Belleville, Ontario, Canada) and were transported to the Department of Biology at Queen's University where they were maintained in dechlorinated freshwater tanks. The fish were acclimated at 9–11 °C for 1 month prior to experiments.

The trout were anaesthetized in a buffered 3-aminobenzoic acid ethyl ester (MS-222, Sigma):fresh water mixture (1:10 000), and the dorsal aorta was cannulated using the method of Smith and Bell (1964). Following surgery, the fish recovered overnight in darkened acrylic boxes supplied with aerated, flowing water at 10 °C.

Blood collection

Approximately 5–6 ml of blood was removed from the dorsal aortic cannula of the fish and placed at 10 °C in a round-bottomed flask with approximately 500 µl of physiological saline (in mmol l^{-1}: 124.1 NaCl; 5.1 KCl; 1.9 $MgSO_4$; 1.5 Na_2HPO_4; 11.9 $NaHCO_3$; 1.1 $CaCl_2$; 5.6 glucose) containing 20 mmol l^{-1} EDTA to prevent clotting. Samples of blood (haematocrit approximately 25 %) were placed into round-bottomed flasks and subjected to one of four experimental manipulations.

Series I

Induction temperature: radioisotopic labelling

A sample of 1 ml of whole blood was collected and held at 10 °C for approximately 5 min and then divided between four round-bottomed flasks placed at 10 °C (control), 15 °C, 20 °C or 25 °C. Blood was labelled with a [^{35}S]methionine–cysteine mixture (EXPRESS; DuPont) at 3700 kBq ml^{-1} in 100 µl of physiological saline containing 5 mmol l^{-1} EDTA and equilibrated for 2 h in rotating, aerated flasks. The 15, 20 and 25 °C blood samples were then transferred back to control temperature (10 °C), equilibrated for 1 h and sampled. Preliminary experiments indicated that 2 h was sufficient for Hsp70 production and isotope incorporation in these cells. Preliminary data also showed that the sampling time of 1 h post-heat-shock was appropriate since the density of all bands on the fluorographs corresponding to Hsp70 remained unchanged for at least 5 h after the heat shock.

Preparation of blood samples for analysis by fluorography consisted of washing each pool of blood once in physiological saline containing 5 mmol l^{-1} EDTA and resuspending the resultant red blood cell pellet in 1 ml of this saline. The suspension was then added to a hypo-osmotic saline (10 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} Tris–HCl; pH 8) containing 10 µl of an ethanol-based protease inhibitor cocktail (0.67 mg ml^{-1} each of phenylmethylsulphonylfluoride, pepstatin A and chymostatin, and 4.7 mg ml^{-1} of *N*-tosyl-L-phenylalanine chloromethyl ketone) and 10 µl of a water-based protease inhibitor cocktail (1.0 mg ml^{-1} each of leupeptin, phenathroline, aprotinin and trypsin inhibitor, and 3.0 mg ml^{-1} of benzamidin). To ensure complete lysis of the red blood cells, the suspension was frozen in liquid nitrogen, then quickly thawed. The resultant lysed cell suspension was spun for 1 h at 42 000 ***g*** in a SW-28 swinging-bucket rotor on a Beckman L8-55M ultracentrifuge thermostatted to 10 °C. The supernatant was then concentrated using a Centricon-10 (Amicon) and a Beckman model J2-21M induction drive centrifuge, thermostatted to 10 °C.

The resultant samples were assayed for total protein concentration using the bicinchoninic acid (BCA) method (Pierce) and the remaining sample was added to an equal volume of sample buffer [in mmol l^{-1}: 40 sodium dodecyl sulphate (SDS), 1 bromophenol blue, 200 glycerol, 100 β-mercaptoethanol, 50 Tris] and frozen at –20 °C overnight.

Electrophoresis

Samples were loaded to equivalent amounts of protein on one-dimensional SDS–polyacrylamide (10 %) gels and subjected to electrophoresis (Laemmli, 1970). The gels were impregnated with EN^{3}HANCE (DuPont/NEN) according to the manufacturer's instructions, dried and subjected to fluorography at –80 °C.

Oxygen consumption

2–3 ml of whole blood was placed into each of two round-bottomed flasks and equilibrated for 2 h at 10 °C (control) or 25 °C (heat shock) in rotating aerated flasks. The heat-shocked blood was then transferred back to 10 °C. Red blood cell oxygen consumption was then determined by incubating a 1 ml sample of whole blood from each flask in a gas-tight syringe and measuring the oxygen content and haemoglobin

concentration over a 5 h period at 10 °C. Oxygen content was measured on 50 µl blood samples using the Tucker method (Tucker, 1967).

Series II

Western blot

A 2 ml sample of whole blood was divided between two round-bottomed flasks and equilibrated for 2 h at 10 °C (control) or 25 °C (heat shock) in rotating aerated flasks. The heat-shocked blood was then transferred to 10 °C, and both pools of blood were further equilibrated for 1 h. Blood was sampled as described in series I.

Equal amounts of protein were loaded onto each sample well of a SDS–polyacrylamide (10 %) gel and separated electrophoretically (Laemmli, 1970). After electrophoresis, gels were soaked for 20 min in transfer buffer (in mmol l^{-1}: 25 Tris, 192 glycine, 1.75 SDS, 20 % v/v methanol). Proteins were then electroblotted onto nitrocellulose (Schleicher and Schuell) using a Bio-Rad mini trans-blot electrophoretic transfer cell. The protein blots were blocked for 1.5 h in 2 % powdered milk in Tris-buffered saline (TBS) (100 mmol l^{-1} Tris, pH 7.5; 150 mmol l^{-1} NaCl) containing 0.05 % Tween 20 (TBS-T). The blots were then reacted with a rainbow trout polyclonal anti-rabbit Hsp70 antibody (gift from Dr G. Iwama, University of British Columbia, Canada) for 1 h and washed three times in TBS-T. This antibody will cross-react with both the constitutive and stress-inducible forms of Hsp70. Following the washes, the blot was reacted with a 1:7500 dilution of the secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG) for 1 h, washed in TBS-T and TBS, and visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sambrook *et al.* 1989).

Series III

Actinomycin D and cycloheximide: radioisotopic labelling

A 4 ml sample of blood was divided equally among four round-bottomed flasks kept at 10 °C. The transcriptional inhibitor actinomycin D was added to one flask to a final concentration of 20 µg ml^{-1}. Preliminary experiments were also conducted with 10 µg ml^{-1} actinomycin D. Cycloheximide, a protein synthesis inhibitor, was added to another flask to give a final concentration of 100 µg ml^{-1}. Preliminary dose–response experiments indicated that 20 µg ml^{-1} actinomycin D and 100 µg ml^{-1} cycloheximide were the most effective doses. All flasks were then equilibrated at 10 °C for an additional 10 min. Next, one flask was kept at 10 °C (control), while the other three, including the two drug treatments, were placed at 25 °C. All flasks were labelled with a 3700 kBq ml^{-1} mixture of [^{35}S]methionine and [^{35}S]cysteine in 100 µl of physiological saline, then aerated and rotated at their respective temperatures for 2 h. Following this equilibration period, all the flasks were returned to 10 °C, equilibrated under the same conditions for 1 h, and then sampled as described in series I. Electrophoresis was performed as outlined in series I.

Series IV

Heat shock and anoxia: experimental protocol

As in series III, a 4 ml sample of blood was divided equally among four round-bottomed flasks. Two flasks were placed at 10 °C (control) and two flasks were placed at 25 °C (heat shock). Each pool of blood was labelled with 3700 kBq ml^{-1} [^{35}S]methionine–cysteine in 100 µl of physiological saline. One flask from each temperature was gassed with 100 % nitrogen and the other with air for the entire experiment. The blood samples warmed to 25 °C were then placed at 10 °C for 1 h. Blood sampling and protein electrophoresis were performed as outlined in series I. An additional experiment was also performed as described above except that recovery took place in air for all treatments.

It should be noted that separate experiments were conducted in both series III and IV to determine whether suspension of the cells in either plasma or physiological saline had any effect on the nature of the observed responses. Since no effect was evident, the data from both experiments were combined.

Quantification and statistical analysis of Hsp70

Quantification of Hsp70 was performed using a Molecular Dynamics computing densitometer with associated Molecular Dynamics ImageQuant (version 3.3) software. In order to correct for the variation among fish and among fluorograms or western blots (due to varying amounts of radioactivity or protein, and exposure times), the stain densities of each fluorogram or blot were normalized to the density of the Hsp70 band from the control lane (10 °C) of each experiment. The density of the Hsp70 band of the control lane was given a value of 1, and the densities of the Hsp70 band from experimental treatments were expressed relative to the control value (Dietz and Somero, 1992). In order to be certain that the increased levels of stress proteins were due to heat shock and not to possible Q_{10} effects, values for control and stressed tissue were also normalized to changes in synthesis of a non-stress protein, actin (approximately 46 kDa). One-way analysis of variance (ANOVA) was used to determine significant (P<0.05) differences in Hsp70 and actin synthesis among temperatures in series I and among treatments in series IV. A t-test was used to test for significant (P<0.05) differences between control and heat-shock temperatures in the oxygen consumption experiments and in series II. In series III, a two-way ANOVA was used to determine the significance (P<0.05) of temperature and experimental treatment on Hsp70 and actin synthesis. A one-way ANOVA was then performed to determine significant (P<0.05) differences between treatments.

Results

Fig. 1 illustrates the effect of temperature on protein synthesis in rbcs from fish acclimated to 10 °C. This fluorogram shows increases in labelling of apparent stress proteins with molecular masses of 70 and 90 kDa at 25 °C. We are confident that the cells survived the heat shock because of

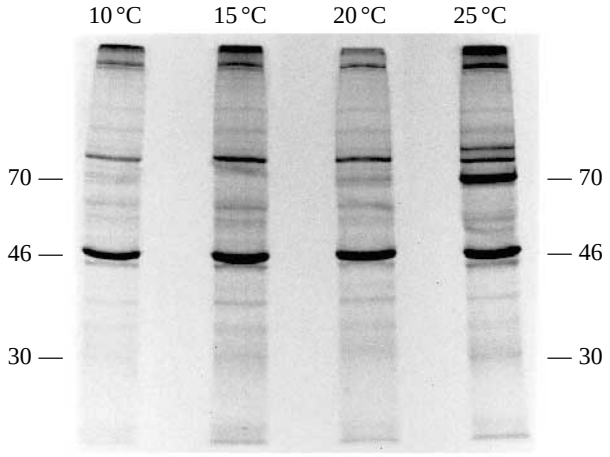

Fig. 1. Representative fluorogram demonstrating the induction and synthesis of Hsp70 from rainbow trout red blood cells labelled with [^{35}S]methionine–cysteine. Red blood cells were equilibrated at 10, 15, 20 and 25 °C for 2 h and then allowed to recover for 1 h at 10 °C. Molecular masses are indicated in kDa.

their ability to synthesize new proteins and because, during the recovery period at 10 °C, there was no significant difference in the oxygen consumption between control (10 °C) cells and cells that had been heat-shocked at 25 °C for 2 h (*N*=6, data not shown). We are also confident that the protein bands represent newly synthesized proteins because we were successful in completely blocking this protein synthesis with cycloheximide,

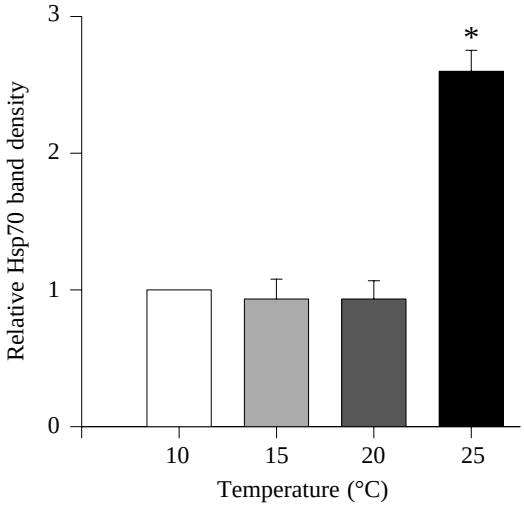

Fig. 2. Quantification of Hsp70 synthesis in rainbow trout red blood cells equilibrated at 10, 15, 20 and 25 °C. Values represent relative Hsp70 band density obtained from densitometric scans of fluorograms from experiments conducted on three individuals. The density of the Hsp70 band from the control lane (10 °C) was given a value of 1, and the densities of the Hsp70 bands from experimental treatments are expressed relative to the control value. All tissue was also normalized to changes in the synthesis of a non-stress protein, actin (approximately 46 kDa). The asterisk indicates a significant difference (P<0.05) from the control condition (10 °C). All values are expressed as mean + S.E.M.

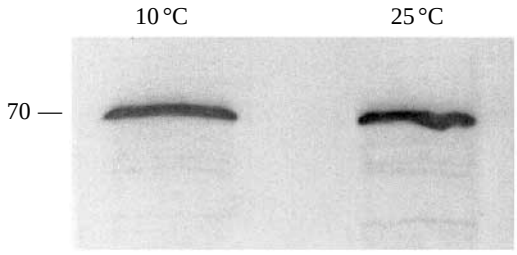

Fig. 3. Representative western blot analysis of Hsp70 from rainbow trout red blood cells equilibrated at 10 °C (control) and 25 °C (heat shock). Lanes were loaded to equivalent amounts of protein. Molecular mass is indicated in kDa.

a known inhibitor of protein synthesis (*N*=5; data not shown). Although our data suggest that there is more than one heat-induced protein synthesized in these cells, the 70 kDa protein is most consistently expressed and is thus the focus of the present study. The synthesis of the apparent 70 kDa stress protein was not significantly affected by temperature increases of up to 10 °C (Fig. 2); however, there was more than a twofold increase in levels of this protein following a 15 °C increase in temperature to 25 °C.

Western blot analysis using a rainbow trout polyclonal antibody indicated that Hsp70 is indeed present in rainbow trout rbcs (Fig. 3) under both control (10 °C) and heat-shock (25 °C) conditions (Fig. 3). Unlike the fluorograms shown in Fig. 1, densitometric analysis of the western blots showed no significant difference between the relative amount of Hsp70 in control *versus* heat-shock conditions (*N*=5, data not shown).

The transcriptional inhibitor actinomycin D was used to examine the mechanism of gene regulation present in trout rbcs. A representative fluorogram from these experiments is shown in Fig. 4, and the normalized, quantitative data from seven individuals are presented in Fig. 5. As demonstrated

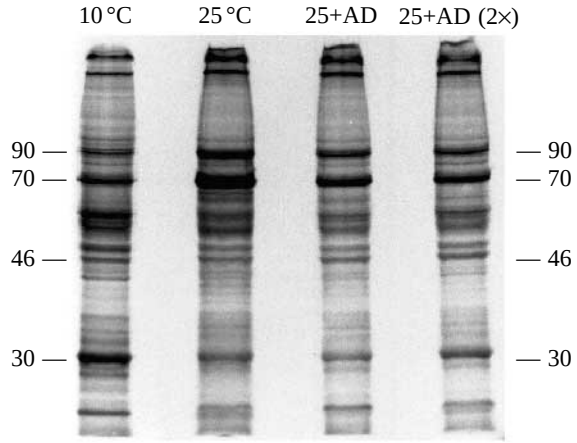

Fig. 4. Representative fluorogram of Hsp70 synthesis in rainbow trout red blood cells labelled with a mixture of [^{35}S]methionine–cysteine and equilibrated at 10 °C and 25 °C, and at 25 °C in the presence of 10 μg ml^{-1} (25+AD) or 20 μg ml^{-1} [25+AD (2×)] actinomycin D. Cells were equilibrated under these conditions for 2 h and then transferred to 10 °C for 1 h. Molecular masses of proteins are indicated in kDa.

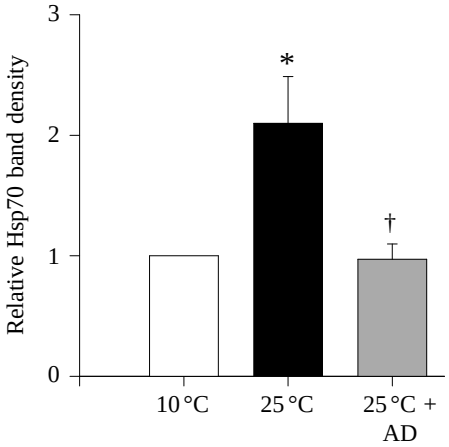

Fig. 5. Quantification of Hsp70 synthesis in rainbow trout red blood cells equilibrated at 10 °C and 25 °C, and at 25 °C in the presence of 20 μg ml^{-1} actinomycin D (AD). The bar graph depicts relative Hsp70 band density obtained from densitometric scans of fluorograms from seven individuals. Relative Hsp70 band density was calculated as described in Fig. 2. The asterisk indicates a significant difference (P<0.05) from the control condition (10 °C) and the dagger denotes a significant difference from the heat-shock condition (25 °C). All values are expressed as mean + S.E.M.

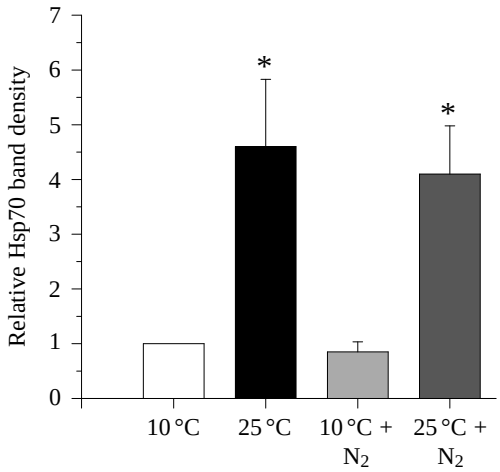

Fig. 6. Quantification of Hsp70 synthesis in rainbow trout red blood cells labelled with a mixture of [^{35}S]methionine–cysteine and equilibrated for 2 h at 10 °C and 25 °C in 100 % air (10 °C and 25 °C) or 100 % nitrogen (10 °C + N_2 and 25 °C + N_2). The cells recovered for 1 h at 10 °C in either air (10 °C and 25 °C) or nitrogen (10 °C + N_2 and 25 °C + N_2). Values represent relative Hsp70 band density obtained from densitometric scans of fluorograms from four individuals. Relative Hsp70 band density was calculated as described in Fig. 2. The asterisks indicate significant differences (P<0.05) from the control condition (10 °C). All values are expressed as mean + S.E.M.

previously, a 25 °C incubation temperature resulted in a significant increase in the relative amount of Hsp70 synthesized and no significant change in the synthesis of actin (a representative control protein located at approximately 46 kDa). Moreover, rbcs that had been pre-treated with actinomycin D and then heat-shocked showed no significant change in control protein synthesis but had a significantly lower relative synthesis of Hsp70 compared with rbcs subjected to the heat-shock treatment alone (Fig. 5).

In order to investigate the importance of oxygen for the relative synthesis of Hsp70 in trout rbcs, we exposed rbcs that had been equilibrated at 10 °C to an increase in temperature (25 °C), to anoxia or to a combination of these two stresses. When the results of these experiments were quantified using densitometry, a significant increase in Hsp70 synthesis following a 2 h heat shock of 25 °C (Fig. 6) was again apparent. Anoxia alone, however, had no effect on Hsp70 (Fig. 6) or actin (data not shown) synthesis. Following exposure to a combined anoxia/heat stress, rainbow trout rbcs exhibited a significant increase (compared with control conditions) in the amount of Hsp70 synthesized that was similar in magnitude to that produced in response to heat stress alone.

We also examined whether the availability of oxygen during the recovery process limited the synthesis of stress proteins in cells exposed either to anoxia alone or to a combination of anoxia and heat shock. The addition of oxygen during this final recovery phase, however, did not significantly alter the responses of cells exposed to these stresses (Fig. 7).

Discussion

The rbcs of fish, like those of other non-mammalian vertebrates, retain their nucleus, mitochondria and other cytoplasmic organelles (Weber, 1982; Schweiger, 1962; Speckner *et al.* 1989) and are capable of transcription and translation. Speckner *et al.* (1989) have demonstrated that fish rbcs synthesize haemoglobin while circulating in the peripheral blood; however, there has been very limited information on the ability of fish rbcs to synthesize other proteins (Koban *et al.* 1991). Our experiments show that circulating rainbow trout rbcs synthesize a range of proteins, including 30, 46, 70 and 90 kDa proteins, as demonstrated by the large amount of ^{35}S incorporated into protein during the experiments (Fig. 1). Thus, fish rbcs may serve as a useful system for protein synthesis studies.

Our results indicate that rainbow trout rbcs synthesize Hsp70 in response to temperature stress (Fig. 1). Although a 90 kDa heat-shock protein is also synthesized in these cells, this study focuses on Hsp70 because it has been described as the major stress-inducible protein in rainbow trout cells (Kothary and Candido, 1982) and is most consistently expressed after heat shock. In our experiments, Hsp70 was induced in rainbow trout rbcs after exposure to 25 °C. This heat-shock induction temperature is very similar to that observed in other studies on rainbow trout cells (Kothary and Candido, 1982; Mosser *et al.* 1986; Misra *et al.* 1989). Rainbow trout are known to have a preference for temperatures between 10 and 15 °C (Schurmann *et al.* 1991) with an upper lethal limit of approximately 26 °C (Kaya, 1978). Trout are, therefore, primarily cold-water-adapted fish, but with the recent warming trends in many freshwater areas, it is not uncommon for populations of these

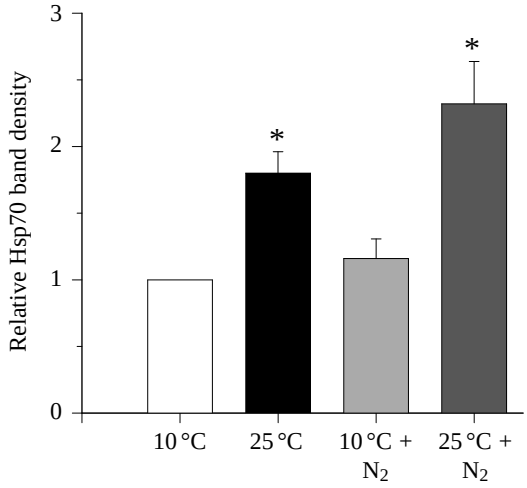

Fig. 7. Quantification of Hsp70 synthesis in rainbow trout red blood cells labelled with a mixture of [^{35}S]methionine–cysteine and equilibrated for 2 h at 10 °C and 25 °C in 100 % air (10 °C and 25 °C) or 100 % nitrogen (10 °C + N_2 and 25 °C + N_2). The cells recovered for 1 h at 10 °C in 100 % air after each treatment. Values represent Hsp70 band density obtained from densitometric scans of fluorograms from five individuals. Relative Hsp70 band density was calculated as described in Fig. 2. The asterisks indicate significant differences (P<0.05) from the control condition (10 °C). All values are expressed as mean + S.E.M.

fish to experience temperatures approaching 25 °C during the summer months. As temperatures approach the upper end of the thermotolerance range of the animal, increased protein misfolding and aggregation occur (Somero, 1995). Recent evidence suggests that the appearance of these misfolded non-native polypeptides is part of the trigger for the stress response (Morimoto *et al.* 1995*b*). Thus, the induction temperature of the heat-shock response may be linked to the thermal stabilities of cellular proteins, as suggested by Dietz and Somero (1993). If this is the case, it seems that rainbow trout rbcs exhibit a high degree of stability until they reach temperatures approaching 25 °C, at which time protein stability is compromised and stress protein synthesis is induced. Acclimation temperature may also influence stress-protein induction temperature in rainbow trout, as has been demonstrated in other species of fish (Dyer *et al.* 1991; Dietz and Somero, 1993; Dietz, 1994). Indeed, the upper lethal temperature for rainbow trout increases as acclimation temperature is increased (Kaya, 1978). Thus, it may be concluded from the present results that rbcs from rainbow trout acclimated to 10 °C have a stress-protein induction temperature of between 21 and 25 °C. Further studies will be required to elucidate the exact nature of the stimulus for stress-protein synthesis in this cell type and to determine whether this induction temperature is influenced by acclimation history.

Western blot data showed no significant difference between the control and heat-shock condition (Fig. 3). Rainbow trout are known to possess at least two heat-shock genes, one stress-inducible (Kothary *et al.* 1984*b*) and one constitutive form (Zafarullah *et al.* 1992). The antibody used in this study does not distinguish between these two forms, but the high levels of Hsp70 protein detected at 10 °C suggest high constitutive levels of Hsp70 in rainbow trout red blood cells. Other studies also confirm that fish heat-shock proteins are expressed in large quantities under non-stressful conditions (Koban *et al.* 1991; Misra *et al.* 1989; Yu *et al.* 1994). In contrast, the radioisotopic labelling experiments do indicate significant differences in Hsp70 synthesis between the control and heat-shock treatments. The radioisotopic data, however, represent relative changes in protein synthesis during a 2 h stress period, whereas the western blot data reflect the total amount of Hsp70 present in the cells. Because of high constitutive levels of Hsp70 in this study, the western blot data probably lack the sensitivity to resolve the increase in Hsp70 synthesis observed during stress. We can be reasonably sure that the identity of the 70 kDa protein in the radioisotopic experiments is Hsp70 because the synthesis of this protein increases dramatically upon heat shock. It should be noted, however, that the western blot data in this study cannot be used to verify or refute conclusively the identity of this radiolabelled 70 kDa protein.

Hsp70 is induced by temperature stresses in other nucleated red blood cells (Banerji *et al.* 1984; Morimoto and Fodor, 1984; Atkinson and Dean, 1985; Winning and Browder, 1988; Koban *et al.* 1991), but the mechanisms involved in the regulation of gene expression in these cells appear to be quite variable. Furthermore, there is a paucity of information on gene regulation of stress proteins in the nucleated rbcs of fish. We found that Hsp70 synthesis in rainbow trout rbcs was inhibited by addition of the transcriptional inhibitor actinomycin D (Figs 4, 5) which inhibits the synthesis of new mRNA. These results indicate that the increased Hsp70 synthesis observed after a heat shock is due to newly transcribed mRNA. This sensitivity of trout rbcs to actinomycin D, coupled with the fact that normal protein synthesis is not affected by heat-shock or stress-protein synthesis, suggests that Hsp70 synthesis in these cells is primarily controlled at the DNA level (transcriptional control). Studies on *Xenopus laevis* and quail (*Coturnix coturnix japonica*) rbcs report similar results (Winning and Browder, 1988; Atkinson and Dean, 1985), whereas Hsp70 synthesis in mummichog (*Fundulus heteroclitis*) and chicken (*Gallus domesticus*) rbcs is thought to be controlled at the RNA level (translational control) (Koban *et al.* 1991; Banerji *et al.* 1984; Morimoto and Fodor, 1984). Kothary and Candido (1982) and Kothary *et al.* (1984*a*) have suggested a complex regulatory mechanism controlling the stress response in rainbow trout cultured cells that involves both transcriptional and translational control. Further studies examining Hsp70 mRNA levels in trout red blood cells may help to resolve this issue.

Proteins are the most energetically expensive molecules to synthesize (Brafield and Llewellyn, 1982) and, in fish, protein synthesis may account for as much as 40 % of the whole-animal oxygen consumption (Lyndon *et al.* 1992). In rainbow trout rbcs, which derive approximately 99 % of their metabolic energy needs from aerobic metabolism (Ferguson *et al.* 1989), one would therefore expect that an aerobic environment might

be crucial for the costly process of protein synthesis. Tufts and Boutilier (1991) showed that exposure to anoxia for 2 h resulted in a 79 % reduction in nucleoside triphosphate (NTP) levels of rainbow trout rbcs, but the effect of oxygen deprivation on cellular protein synthesis has never been investigated in fish. Our results indicate that exposure to anoxia for 2 h had no significant effect on the synthesis of many proteins (e.g. actin) in rainbow trout rbcs. Moreover, these cells were still able to enhance their synthesis of Hsp70 in response to heat stress, regardless of the environmental oxygen levels (Figs 6, 7). It should be noted that previous studies clearly indicate that fish rbcs become anaerobic under these anoxic conditions (Ferguson and Boutilier, 1989; Tufts and Boutilier, 1991). It is possible that the energy deficit incurred during oxygen deprivation in rainbow trout rbcs is largely corrected through anaerobic glycolysis (Pasteur effect) and that this anaerobic contribution is sufficient for continued protein synthesis in these cells (Hochachka, 1986). Conversely, although rainbow trout are sensitive to oxygen, their rbcs may be somewhat tolerant of anoxia (Ferguson and Boutilier, 1989). These cells may be able to preserve the NTP-consuming processes of transcription and translation by adjusting the rate of other NTP-consuming processes, such as ion transport. Although these possibilities cannot be confirmed within the scope of this study, it appears that rainbow trout have evolved strategies that allow them to maintain protein synthesis and the integrity of the heat-shock response in their rbcs even during periods of anoxia. Initially, this apparent anoxia-tolerance seems somewhat surprising since rainbow trout are typically found in well-oxygenated waters. One can speculate, however, that some degree of anoxia-tolerance is a useful trait for red blood cells, which may routinely be exposed to very low oxygen levels during their transit through the capillaries of some tissues (e.g. white muscle) and through the venous circulation, particularly in a highly active fish such as the rainbow trout.

Both hypoxia and anoxia stresses alone have been shown to induce the synthesis of a family of Hsp70 proteins in a variety of tissues (Hammond *et al.* 1982; Sciandra *et al.* 1984; Tuijl *et al.* 1991; Mestril *et al.* 1994; Myrmel *et al.* 1994; Kobayashi and Welsh, 1995). Although rainbow trout rbcs continued to synthesize proteins during anoxia, they did not increase their synthesis of Hsp70 under these conditions (Figs 6, 7). To our knowledge, oxygen deprivation as an inducer of the heat-shock response has been demonstrated chiefly in mammals, and in critical tissues that would be most sensitive to this stress, such as the brain and heart. The reason for the absence of an anoxic stimulus for the synthesis of stress proteins in fish rbcs may again be related to the fact that these cells are frequently exposed to low oxygen levels in the circulation. It is noteworthy that hepatocytes of the western painted turtle (*Chrysemys picta bellii*), an animal able to withstand anoxia, also lacked a heat-shock response after exposure to anoxia (Land *et al.* 1993). It may be energetically costly to increase the synthesis of stress proteins in response to hypoxia/anoxia for cells that are frequently exposed to periods of reduced oxygen availability during routine activities. Moreover, if cells are adapted to hypoxia/anoxia, they may not require the protective, restorative roles of stress proteins under these conditions. The present results indicate that anoxia will not induce heat-shock protein synthesis in rainbow trout rbcs; however, further studies comparing the oxygen sensitivity of stress protein synthesis in anoxia-tolerant and anoxia-sensitive tissues are probably warranted.

We would like to thank Dr George Iwama and Rob Forsyth for donating the trout antibodies. We also thank Dr Chris Moyes for excellent discussions during the course of this work. This study was supported by a Natural Science and Engineering Research Council (NSERC) postgraduate scholarship to S.C. and an NSERC operating grant to B.L.T.

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