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Reduced Enzyme Activity Following Hsp70 Overexpression in *Drosophila melanogaster*

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Acclimation to environmental change can impose costs to organisms. One potential cost is the change in cell metabolism that follows a physiological response, e.g., high expression of heat shock proteins may alter specifi activity of important enzymes. We examined the significanc of this cost in a pair of Drosophila melanogaster *lines transformed with additional copies of a gene that encodes the heat shock protein, Hsp70. Heat shock induces Hsp70 expression in all lines, but lines with extra copies produce much more Hsp70 than do excision control strains. The consequence of this supranormal Hsp70 expression is to reduce specifi activity of both enzymes analyzed, adult alcohol dehydrogenase (ADH), which is heat sensitive, and lactate dehydrogenase, which is not. Strain differences were most pronounced under those conditions where Hsp70 expression was maximized, and not where the heat stress denatured proteins. That result supported the idea that Hsp70 expression is constrained evolutionarily by its tendency to bind nascent peptides when overabundant within the cell.*

KEY WORDS: enzyme activity; Hsp70; *Drosophila melanogaster*; heat shock; metabolism; stress; trade-offs.

INTRODUCTION

Individuals of many species respond physiologically when they encounter a novel thermal environment. Such change, termed thermal acclimation, may enable an organism to survive a stress that might otherwise be lethal. However, thermal

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acclimation is sometimes neutral or even deleterious with respect to fitness (Bennett and Lenski, 1997). Two possible mechanisms may explain such non-adaptive outcomes from expression of a new thermal phenotype (Feder, 1996): one is metabolic, where a response consumes too much energy or nutrients and thereby jeopardizes other cellular functions; the second is biochemical, because the defense proteins interfere with enzymatic processes within the cell.

Almost all organisms respond to heat stress by producing high concentrations of heat shock proteins (Hsp's). In *Drosophila*, Hsp's can be virtually absent from the cell before heat stress and suddenly undergo 10,000-fold increases in expression (Lindquist, 1993). These proteins may accumulate to account for 10–15% of the soluble protein in a cell (Loomis and Wheeler, 1982; Palter *et al.*, 1986), displacing routine protein synthesis. The negative consequences of this intense protein expression for performance and fitness can be profound: *Drosophila* cells that express Hsp's at benign temperatures grow more slowly than normal cells (Feder *et al.*, 1992), a yeast strain that cannot express Hsp104 grows faster than its wild-type counterpart on some media (Sanchez *et al.*, 1992), fecundity declines in adult *D. melanogaster* treated to induce the heat shock response (Krebs and Loeschcke, 1994), and overexpression of Hsp70 reduces the larva-to-adult survival of *D. melanogaster* (Krebs and Feder, 1997a). Heckathorn *et al.* (1996a) also found that, when nitrogen availability is limiting in corn, other proteins may be catabolized to provide amino acids for stress protein production. Consequently, loci such as those that code for Hsp's face antagonistic selection, as the costs of stress tolerance potentially cancel out the benefits of acclimation (Calow, 1991; Coleman *et al.*, 1995; Hoffmann, 1995; Krebs and Loeschcke, 1996; Parsons, 1996).

Negative outcomes of thermal acclimation are consistent with the hypothesis that Hsp's are toxic at very high concentrations (Krebs and Feder, 1997a). To examine this toxicity, we exploited *Drosophila* that had been genetically engineered to overexpress the Hsp, Hsp70 (Welte *et al.*, 1993). Even without this transgene, *Drosophila* larvae express Hsp's massively in response to a mild heat shock, such as those that may occur in nature (Junge-Berberovic, 1996; Feder, 1996; Feder *et al.*, 1997). With the transgene, the extra-copy strain produces at least twice as much Hsp70 as does its control, a strain in which the transgene was excised but which maintains an insert at an identical point in the genome. Two pairs of each strain were available for study.

Here we compared how overexpression of Hsp70 interacts with the function of other enzymes by measuring the specific activity of lactate dehydrogenase (LDH) and adult alcohol dehydrogenase (ADH) following exposure to stress. These enzymes were chosen because LDH is known to be important in thermal adaptation in fish (Powers and Schulte, 1998), and it is thermally stable, while ADH denatures at temperatures within the survival range of flies (Feder and Krebs, 1998). Specific activity was measured for each strain in the absence of stress, after exposure to the temperature that maximally induces Hsp70 in adults (1 hr at 36°C), after exposure

to a near-lethal temperature (1 hr at 38.5°C), and where flies were first pretreated at 36°C, rested for 1 hr, and then exposed to the higher stress.

METHODS

We characterized the effects of varying Hsp70 expression and heat treatments on adults in a pair of strains engineered to vary in *hsp70* copy number and, consequently, expression of Hsp70. Welte *et al.* (1993) described the construction of the transgenic strains via unequal homologous recombination. The extra-copy strain contains a pair of transgene constructs, each a composite of three *hsp70* genes, an eye color marker, w^{hs}, and flanking yeast recombination targets and P-elements. The excision strain shares the same chromosomal site of transgene integration and flanking elements but lacks the *hsp70* transgenes and eye color marker. The procedure that duplicates the initial insert in one chromatid simultaneously deletes the insert in another, thereby producing two strains varying in copy number but possessing similar genetic backgrounds (details given by Feder *et al.*, 1996). One member of the pair, the extra-copy strain, has 12 *hsp70* transgenes in addition to its 10 natural copies (Ish-Horowicz *et al.*, 1979). The other member, the excision strain, has only the 10 natural copies but shares the transgene integration site with the extra-copy strain and, thus, is a control for insertional mutagenesis. For the strains used here, the site of transgene integration mapped to chromosome II in one strain and to chromosome III in the other.

Preliminary assays verified that the lines containing the inserted *hsp70* gene copies (strains TraII and TraIII) still produced two to three times as much Hsp70 as did lines where the genes were excised (CisII and CisIII). Pairs of flies were transferred to microtubes with 10 μl phosphate-buffered saline and treated for 1 hr at 36°C and 1 hr at 25°C, which induces maximal levels of expression of Hsp70 in *D. melanogaster* (Krebs and Feder, 1997a; Krebs, 1999). All flies were first frozen in liquid nitrogen and stored at −80°C prior to the ELISAs. Six or seven pairs per line were analyzed by an enzyme linked immunosorbent assay (ELISA), which has been described elsewhere (Welte *et al.*, 1993; Feder *et al.*, 1996; Krebs and Feder, 1997a).

The enzymes assayed for specific activity were lactate dehydrogenase (LDH), which catalyzes pyruvate to lactate in a reaction that recovers NAD from NADH, and adult alcohol dehydrogenase (ADH), which catalyzes ethanol to acetaldehyde while reducing NAD. Before analysis of the specific activity for LDH and ADH, we exposed adults to one of a series of temperature treatments: either no treatment, pretreatment only (1 hr at 36°C and 1 hr of recovery at 25°C), heat shock only (1 hr at 38.5°C), or pretreatment followed by heat shock. For the ADH study, the recovery time after stress was also varied among groups as 1, 2, or 3 hr. All treatments were performed in water baths accurate to 0.1°C, with flies housed in inverted glass vials under saturated humidity. Subsequent to treatment, pairs of flies

were transferred to 1.5-ml cryotubes, quick-frozen in liquid nitrogen, and stored at −80°C. Assays of specific activity of LDH and ADH were made for pairs of flies.

For LDH determination, flies were homogenized in a 320-μl grinding buffer (100 m*M* Hepes, 10 m*M* KCl, 5 m*M* EGTA, 0.1 m*M* DTT) on ice and then centrifuged for 10 min at 13,000 rpm and 4°C. In triplicate, 60 ml of this homogenate was mixed with 10 μl of assay buffer (100 m*M* Hepes, 10 m*M* KCl, 5 m*M* $MgCl_2$, 3.8 m*M* ADP), 20 μl of 0.4 m*M* NADH, and 10 μl of 15 m*M* pyruvate (Pierce and Crawford, 1994). For ADH, flies were ground in 320 μl of 0.1 *M* Tris–HCl, and 60 μl of the homogenate was mixed with 30 μl of 4 m*M* NAD^+ and 10 μl of 1 *M* ethanol, also in triplicate. The reaction was run at 30°C for 30 min (McKechnie and Geer, 1984). Specific activity was recorded as V_{max} by recording the change in absorbance in a Molecular Devices thermomax plate reader at 340 nm and is expressed as $mOD_{340} \cdot min^{-1}$.

RESULTS

The transgenic manipulation to increase Hsp70 expression did not affect LDH or ADH concentrations in the absence of stress. Also, when a heat stress was applied (1 hr at 38.5°C), specific activity levels of LDH remained similar to those of untreated individuals in all strains (Fig. 1). Thus heat did not denature the LDH. However, specific activity declined in extra-copy individuals exposed to pretreatment alone, which is a mild stress that induces high Hsp expression. Strain differences were significant where individuals received no further stress ($P < 0.05$, as analyzed by strain × treatment interactions in ANOVA or by *t* tests comparing differences between each group).

Stress exposures produced a different picture when a heat-sensitive enzyme was analyzed; ADH levels fell after the heat shock, exposure to 38.5°C for 1 hr, and this effect predominated in females, in which the specific activity was always much lower than in males (Fig. 2). Like the results for LDH, the high Hsp70 levels induced by pretreatment further reduced the specific activity of ADH in females, but in contrast, enzyme activity in the males changed little (strain × gender interaction, $P < 0.001$). Specific activity in females dropped by a third after heat shock and then largely recovered over a 3-hr period in the excision lines but not in the extra-copy lines (for expression 3 hr after heat shock: effect of gender, $P < 0.001$; strain × gender, $P < 0.05$). Both males and females recovered activity faster if pretreated than if exposed directly to 38.5°C without pretreatment, even though the decline in activity from pretreatment and heat shock exceeded the loss from heat shock alone. Females of the extra-copy strain failed to return ADH activity to control levels within the short duration of the study, whether pretreated ($P < 0.05$) or not ($P < 0.01$). ADH activity of males after 3 hr, however, did not differ significantly from untreated levels.

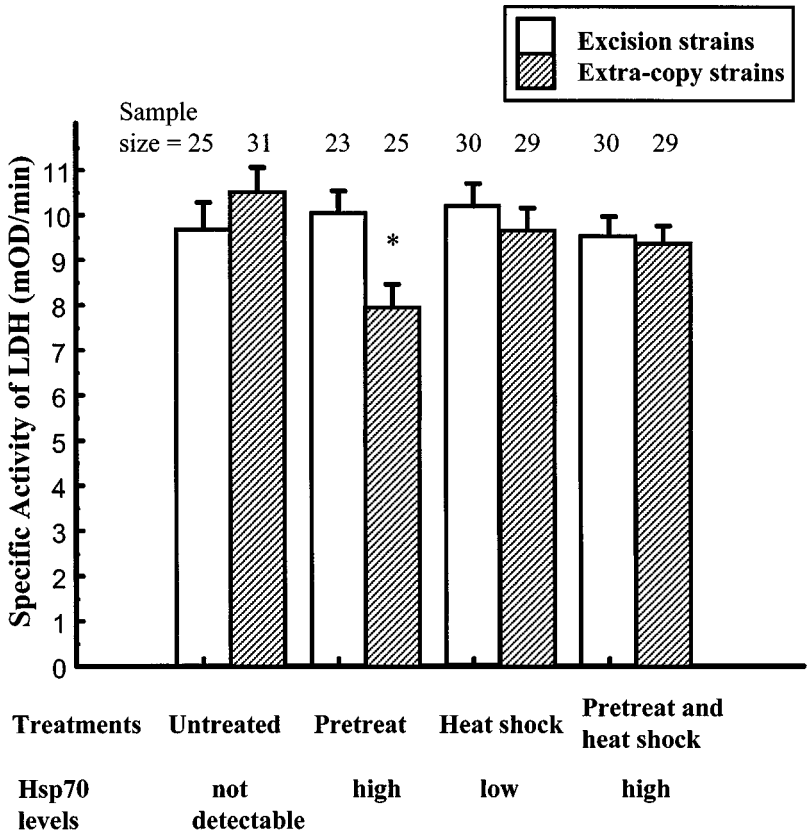

Fig. 1. The specific activity of lactate dehydrogenase (LDH) in *D. melanogaster* adults from the extra-*hsp70*-copy strains and the strains from which the inserted copies were excised. Activity of LDH declined only when flies of the extra-copy strain were treated to induce maximal Hsp70 expression (after pretreatment) without any subsequent heat shock. Variation among lines in other treatment groups, including increased heat, was not significant. Sample sizes are indicated above each bar (±1 SE).

DISCUSSION

Thermal stress causes many physiological changes (Huey and Bennett, 1990; Feder, 1996), of which one is expression of chaperones like Hsp70 that assist cells to repair damage after protein denaturation and aggregation. Organisms require this additional Hsp70 expression to survive stress, but the amount produced must balance the beneficial and negative consequences that occur when these binding proteins occur in high concentrations (Krebs and Feder, 1998a). Here we found that overexpression of Hsp70 can reduce the specific activity of normal cell enzymes like ADH and LDH.

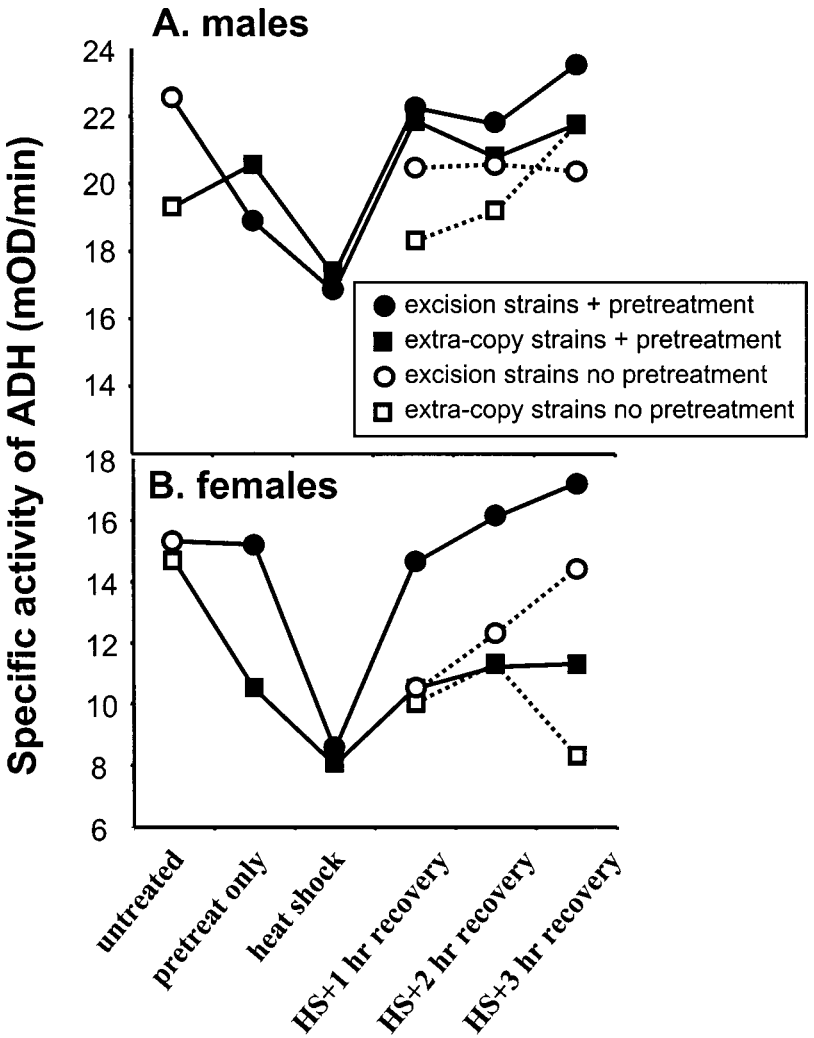

Fig. 2. The specific activity of alcohol dehydrogenase (ADH) in *D. melanogaster* males (A) and females (B) from the extra-*hsp70*-copy strains and the strains from which the added copies were excised. Assays are shown for a time course of treatments: flies that were untreated (controls), pretreated at 36°C to induce high Hsp70 expression, and heat-shocked at 38.5°C for 1 hr, either not pretreated or pretreated and then heat-shocked before they were allowed to recover at 25°C for 1, 2, or 3 hr. Almost all standard errors were small and ranged between 1 and 2 mOD/min; the exception was males pretreated and heat-shocked without recovery. On average, each of the 36 means was derived from about 10 replicates: range, $N = 5$ to 22 across each combination of gender, strain, and treatment.

Previously, costs were found at a phenotypic level, as changes in cell growth or survival: high concentrations of Hsp70 and other 70-kD family members are sufficient to raise mortality (Feder *et al.*, 1992; Krebs and Feder, 1997a), inhibit protein secretion (Dorner *et al.*, 1988, 1992), and promote protein aggregation *in vitro*, (M. J. Borrelli and J. Lepock, personal communication). Therefore, while raising Hsp70 levels improves stress tolerance (Feder *et al.*, 1996; Hightower *et al.*, 1999), these fitness benefits after stress may trade off with costs in its absence (Krebs and Feder, 1997b). As a consequence, some natural populations from polluted environments may express lesser amounts of Hsp's than will individuals from clean habitats, such as snails (Tomanek and Somero, 1999) and soil arthropods (Köhler *et al.*, 2000).

Evidence for a mechanism, however, is weak, although two hypotheses can explain fitness decline where a protein is overexpressed. First is an increase in energetic expenditure (Koehn and Bayne, 1989; Koehn, 1991; Hawkins, 1991). Predictions generated from this hypothesis of metabolic cost, for example, lower fitness when an organism overexpresses a benign protein, are not supported for *Drosophila* (Krebs and Feder, 1998b). The second hypothesis is for an interference model, a biochemical cost; high concentrations of Hsp70 may preferentially bind denatured proteins, but when none are available, binding may occur with nascent peptides in the cell. If true, the specific activity of randomly assayed enzymes from homogenates will be lower in the presence of high quantities of Hsp70 than when Hsp70 is at normal or lower levels. This prediction, although an indirect test of binding, was met.

Support for an interference model of costs with Hsp70 overexpression derives from a second predicted effect. Given that pretreatment alone reduces the activity of a heat-tolerant enzyme, a strong stress that can denature some cellular proteins should return the activity of this enzyme to normal levels. After exposure to the higher stress, LDH activity returned to normal levels even in extra-copy flies. Direct exposure to a high stress alone, however, should not reduce enzyme activity, because Hsp70 in *D. melanogaster* is poorly induced at temperatures above 37°C (Krebs and Feder, 1997a).

Two other studies obtained results that also are consistent with biochemical costs from Hsp expression. First, Su *et al.* (1999) observed a reduced release of LDH in heart myoblasts that overexpress Hsc70, a protein similar to Hsp70 in structure, but it is not stress regulated. Second, Heckathorn *et al.* (1996a,b) proposed that allocation of resources under stress sets a conflict between amino acids required for Hsp's and other protein synthesis when nutrients are especially limiting. When assaying corn over many hours, they found that rubisco and pepcase levels fell after heat shock and that the effects increased under low nitrogen. However, these results can occur either because the cell actively degraded protein to satisfy nutrient need, the explanation proposed, or because the protein was initially, and possibly inappropriately, bound by the Hsp's and was consequently degraded.

One function of Hsp's is to target damaged proteins for the ubiquitin degradation pathway (Niedzwiecki and Fleming, 1993; Luders *et al.*, 2000).

Because Hsp's can affect cells in different ways, definitively identifying the predominant mechanism that explains the recovery of enzyme activity is difficult in whole organisms. We suggest that Hsp70, when superabundant, reduces the specific activity of LDH and ADH directly by binding them, because activity usually recovers rapidly in lines where expression is normal. Activity recovered fully after a severe stress in all groups except for ADH in females of the extra-copy lines. No explanation for this exception is apparent. However, in all groups including this one, activity for flies pretreated and stressed equaled or exceeded that of flies heat-shocked without pretreatment, confirming the requirement for the heat shock response to protect enzyme activity and cell function. New enzyme synthesis cannot explain this recovery, because a coordinate depression in the synthesis of proteins other than Hsp's occurs during heat shock in *Drosophila*. Recovery of synthesis requires 2–3 hrs after a stress abates (DiDomenico *et al.*, 1982; Solomon *et al.*, 1991).

In summary, our results add to the growing work on the evolutionary consequences of variation in Hsp70 and inducible thermotolerance by providing additional evidence that the heat shock response has costs, and suggests that excessive Hsp70 levels can interfere with normal cell function. Raising Hsp70 levels is an important part of thermotolerance, but fitness benefits after stress may trade off with costs of induction when stress levels are low.

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