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### Research Article

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## Induction of Stress Proteins in Cultured Myogenic Cells

### Molecular Signals for the Activation of Heat Shock Transcription Factor during Ischemia

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#### Abstract

Expression of major stress proteins is induced rapidly in ischemic tissues, a response that may protect cells from ischemic injury. We have shown previously that transcriptional induction of heat-shock protein 70 by hypoxia results from activation of DNA binding of a preexisting, but inactive, pool of heat shock factor (HSF). To determine the intracellular signals generated in hypoxic or ischemic cells that trigger HSF activation, we examined the effects of glucose deprivation and the metabolic inhibitor rotenone on DNA-binding activity of HSF in cultured C2 myogenic cells grown under normoxic conditions. Whole-cell extracts were examined in gel mobility shift assays using a 39-bp synthetic oligonucleotide containing a consensus heat-shock element as probe. ATP pools were determined by high-pressure liquid chromatography and intracellular pH ( $pH_i$ ) was measured using a fluorescent indicator. Glucose deprivation alone reduced the cellular ATP pool to 50% of control levels but failed to activate HSF. However,  $2 \times 10^{-4}$  M rotenone induced DNA binding of HSF within 30 min, in association with a fall in ATP to 30% of control levels, and a fall in  $pH_i$  from 7.3 to 6.9. Maneuvers (sodium propionate and amiloride) that lowered  $pH_i$  to 6.7 without ATP depletion failed to activate HSF. Conversely, in studies that lowered ATP stores at normal pH (high  $K^+$ /nigericin) we found induction of HSF-DNA binding activity. Our data indicate that the effects of ATP depletion alone are sufficient to induce the DNA binding of HSF when oxidative metabolism is impaired, and are consistent with a model proposed recently for transcriptional regulation of stress protein genes during ischemia. (J. Clin. Invest. 1992; 89:1685-1689.) Key words: acidosis • gene regulation • heat shock factor • ischemia • stress proteins

#### Introduction

A variety of noxious stimuli, including hypoxia or ischemia, trigger the rapid synthesis of several highly conserved proteins (heat-shock proteins [HSP])<sup>1</sup> (1, 2). The apparent cytoprotective

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1. Abbreviations used in this paper: BCECF, 2',7'-biscarboxyl-ethyl-5(6)-carboxyfluorescein; HSE, heat-shock element; HSF, heat-shock transcription factor; HSP, heat-shock protein.

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effects of these proteins during thermal stress have stimulated interest in a potentially beneficial role for stress proteins during or subsequent to myocardial ischemia (3-5). Much attention has been focused on the major heat-shock genes (HSP70), which comprise some of the best-studied models of inducible promoters in eukaryotes (6, 7). Transcriptional activation of the human HSP70 promoter requires the post-translational activation of the heat-shock transcription factor (HSF) to promote its binding to the five-base repeat—NGAAN—or heat-shock element (HSE) (8, 9). However, the proximate stimuli by which diverse stresses such as heat-shock and ischemia induce the expression of stress protein genes remain incompletely understood (10, 11).

Our laboratory has undertaken studies to determine the intracellular mechanisms by which HSP70 gene transcription is induced in ischemic cells (12, 13). During ischemia, the heat-shock response is triggered and the subsequent synthesis of stress proteins probably contributes to the restoration of cellular homeostasis after reperfusion (3). In a previous publication, we have shown that transcriptional induction of an HSP70 gene by hypoxia, like heat shock, is attributable to activation of DNA-binding of HSF (12). Hypoxic activation of HSF may be a direct result of reduced oxygen tension, perhaps a conformation change in an oxygen binding protein, or an indirect effect triggered by metabolic sequelae of hypoxia, such as ATP depletion and/or acidosis. Recent studies in vitro by Mosser et al. (14) suggest that the denaturing effects of low  $pH_i$ , detergents, or urea can influence the DNA-binding activity of HSF. In the present study, we have compared the effects of metabolic inhibition by rotenone, glucose deprivation, and intracellular acidification by propionic acid and amiloride on the DNA-binding activity of HSF in C2C12 myogenic cells.

#### Methods

**Cell culture conditions.** C2C12 myogenic cells ( $1 \times 10^6$  cells) were plated in DME supplemented with 10% (vol/vol) fetal calf serum in Corning dishes (100 mm) or T75 flasks in a humidified atmosphere of 95% air/5%  $CO_2$ . For cytoplasmic pH measurement, the cells were grown to equivalent confluence on glass coverslips and all experiments were performed 2 d later. To study the effects of metabolic inhibition, rotenone (Sigma Chemical Co., St. Louis, MO) was first dissolved in chloroform (0.001% vol/vol) and stirred into glucose-free DME ( $2 \times 10^{-4}$  M) for 15 min. All control studies were performed with glucose-free DME and included the vehicle, chloroform (0.001% vol/vol). These experiments were performed at 37°C and included preheating all solutions to 37°C before use. For heat-shock conditions, sealed flasks were incubated at  $42 \pm 0.2^\circ C$  for 15 min and harvested immediately for whole-cell extracts as noted below.

**Analysis of HSF binding activity.** Whole cell extracts were prepared as described previously (12). In brief, the cells were pelleted and resuspended in 1.5 vol of lysis buffer (10 mM Hepes, pH 7.9/10 mM KCl/1.5 mM  $MgCl_2$ /0.4 mM sodium orthovanadate/0.4 mM NaF/0.5 mM

PMSF/1.0 mM dithiothreitol) and allowed to stand on ice for 20 min. To each sample 1.6 vol of extraction buffer (20 mM Hepes, pH 7.9/0.2 mM EDTA/20% [vol/vol] glycerol/1.6 M KCl/0.4 mM sodium orthovanadate/0.4 mM NaF/0.1 mM PMSF/1.0 mM dithiothreitol) was added and extraction carried out for 1 h at 4°C with constant shaking. After 100,000 g centrifugation for 1 h, the supernatant was dialyzed for 4 h in 20 mM Hepes, pH 7.9/0.1 mM EDTA/10% (vol/vol) glycerol/50 mM KCl/0.4 mM sodium orthovanadate/0.4 mM NaF/0.1 mM PMSF/1.0 mM dithiothreitol. Protein concentrations were determined in a reagent assay (Bio-Rad Laboratories, Richmond, CA).

To determine the HSF-binding activity 10- $\mu$ g cell extracts were analyzed in gel-mobility shift assays using the double-stranded HSE oligonucleotide (AATTCGAAACCCCTGGAATTTCCGACCTGGC-AGC and its complementary strand) labeled with  $^{32}$ P filling in 5' overhangs with Klenow polymerase (15). After incubation in binding buffer (20 mM Hepes, pH 7.9/40 mM KCl/1.0 mM MgCl<sub>2</sub>/0.1 mM EGTA/0.5 mM dithiothreitol) for 20 min at room temperature, the samples were loaded onto 4% polyacrylamide gels and electrophoresed for 2 h at 200 V. The gel was dried under vacuum and exposed to Omat film (Eastman Kodak Co., Rochester, NY) at -70°C.

**Measurement of cytoplasmic pH ( $pH_i$ ).** The pH-sensitive dye, 2',7'-biscarboxyl-ethyl-(5(6)-carboxyfluorescein (BCECF) was used for the continuous recordings of  $pH_i$ . 2 d after plating, glass coverslips containing C2C12 myogenic cells were rinsed twice with DME solution without glucose or phenol red and loaded with BCECF-AM (10  $\mu$ M) for 30 min at 37°C. The coverslips then were placed into plastic cuvettes and positioned at a 30° angle to the excitation beam of a spectrofluorometer (model 8000C, SLM Instruments, Inc., Urbana, IL) (16). Cells were continuously perfused at 20 ml/min with solutions equilibrated with 5% CO<sub>2</sub> at 37°C. For measurement of  $pH_i$ , the ratio of fluorescence with 500- and 450-nm excitation, 530-nm emission, was corrected for background fluorescence obtained before dye loading (17). The calibration of BCECF was determined using the high K<sup>+</sup>/nigericin technique (17). Cells were loaded with BCECF and transferred into calibration solutions containing 120 mM KCl, 1.54 mM MgCl<sub>2</sub>, 1.1 mM CaCl<sub>2</sub>, 30 mM Hepes, and 10  $\mu$ g/ml nigericin (a K/H antiporter), and the pH varied from 6.4 to 7.6 at 37°C with *N*-methyl-D-glucamine.

**Measurement of intracellular ATP pools.** After the test period, the cells were immediately placed on ice and all subsequent steps were performed at 4°C. The plates were rinsed twice with ice-cold 1 × PBS and the pellets were resuspended in 60% methanol and stored at -70°C until further analysis by high-pressure liquid chromatography. Cell pellets were extracted overnight at -20°C with 1 ml of 60% methanol. Extracts were clarified by centrifugation at 4°C in a microcentrifuge and supernatants were collected. The pellets were reextracted with an additional 1 ml of ice-cold 60% methanol and centrifuged as above and the extracts were combined. The extracts were then dried in vacuo, and the residues were resuspended in 100  $\mu$ l of H<sub>2</sub>O. 50- $\mu$ l aliquots were fractionated with anion exchange chromatography as noted below. A Partisil 5 SAX column (Whatman Inc., Clifton, NJ) was used in conjunction with a GAX-013 guard cartridge (Brownlee Labs, Santa Clara, CA). Chromatography was performed at ambient temperature with gradient elution at a flow rate of 1 ml/min, using the following mobile phases: mobil phase A = 0.5 mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, pH 2.8; mobil phase B = 0.75 M NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, pH 3.5. From the initial conditions (100% A) a linear gradient was run in 35 min to 100% B. This final condition was maintained for 8.5 min followed by return to initial conditions over 1 min. The column was then reequilibrated for 15 min before the next injection. The column effluent was monitored at 260 and 280 nm and adenine nucleotides were quantified by comparing their peak areas to those of external standards.

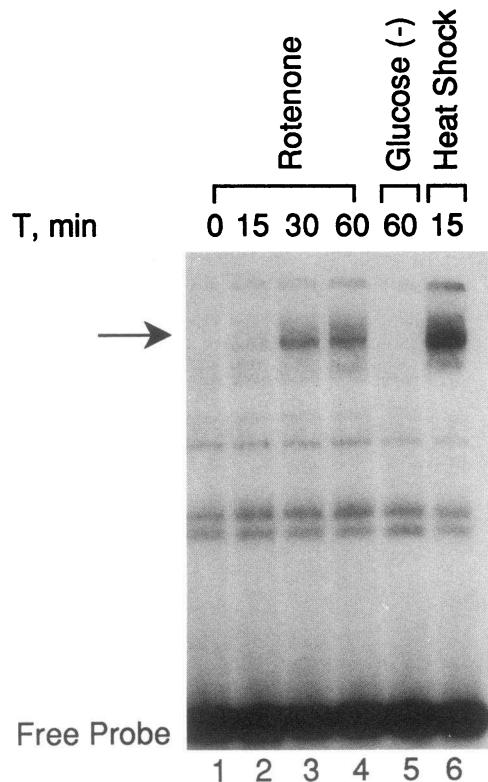
## Results

**Rotenone, a potent inhibitor of mitochondrial oxidative phosphorylation, induces specific DNA-binding activity in C2C12 myogenic cells under normoxic conditions.** In gel mobility shift assays of whole-cell extracts after rotenone treatment, HSF-

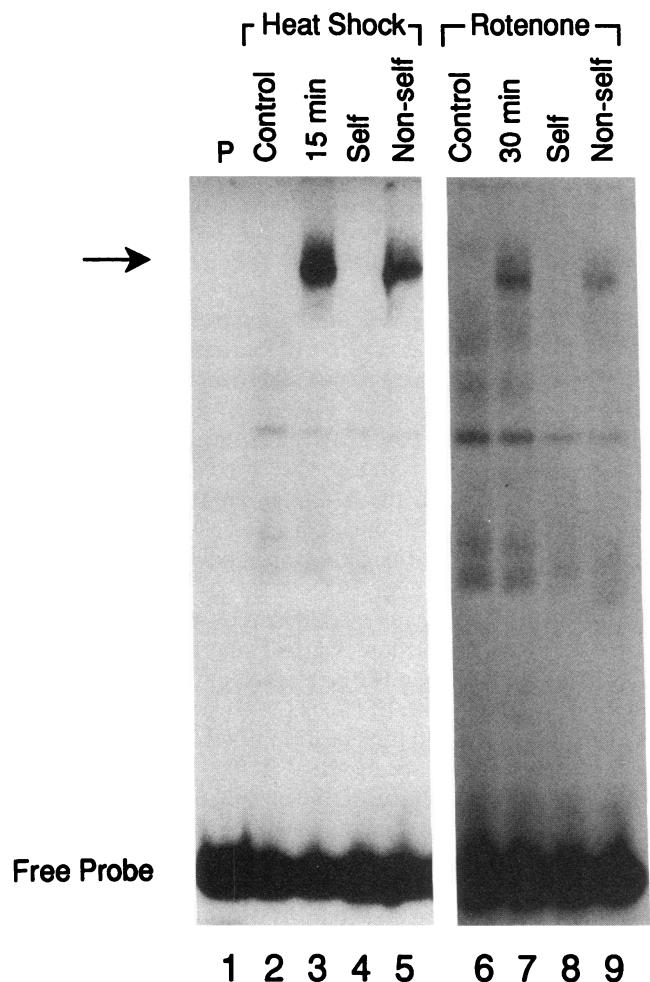
DNA binding activity was induced within 30 min (Fig. 1, lanes 1-4). Similar binding activity was present after 15 min of heat shock (Fig. 1, lane 6) but was not evident in cells incubated for 60 min in glucose-free DME in the absence of rotenone (Fig. 1, lane 5). The specificity of binding was determined by competition with a 200-fold molar excess of unlabeled self or non-self oligonucleotides (Fig. 2). We observed complete competition of binding activity in the presence of excess unlabeled HSE oligonucleotide (self) but not in the presence of a mutant HSE (non-self), confirming that the specificity of HSF-DNA binding activity during heat-shock and rotenone treatments was similar (Fig. 2). These findings suggest that HSF activation during hypoxia occurs as a result of the metabolic consequences of hypoxia, rather than from a direct oxygen-sensing mechanism. Such consequences may include ATP depletion or acidosis from the production of lactate as a result of inhibition of oxidative metabolism.

By measurement of the excitation ratio of intracellular BCECF, we found that severe cellular acidosis was induced by rotenone treatment within minutes. At 30 min, when specific binding activity of HSF was clearly detectable,  $pH_i$  had fallen to approximately 6.9 (Fig. 3). Over the same period, cells incubated in glucose-free medium in the absence of inhibitor maintained  $pH_i$ , near to control levels, and failed to activate the DNA-binding activity of HSF (Figs. 1 and 3).

Rotenone also induced a precipitous fall in ATP concentra-



**Figure 1.** Effects of metabolic inhibitors on DNA-binding activity in protein extracts from cultured C2C12 myogenic cells. Gel retention assays were performed using a synthetic double-stranded oligonucleotide containing a consensus heat-shock element as probe. The position of migration of the unbound free probe in the gel is indicated. Stress-inducible DNA protein complexes (arrow) formed after exposure to rotenone (lanes 1-4) or glucose-free medium in the absence of inhibitor (lane 5) and after heat-shock for 15 min at 42°C (lane 6).

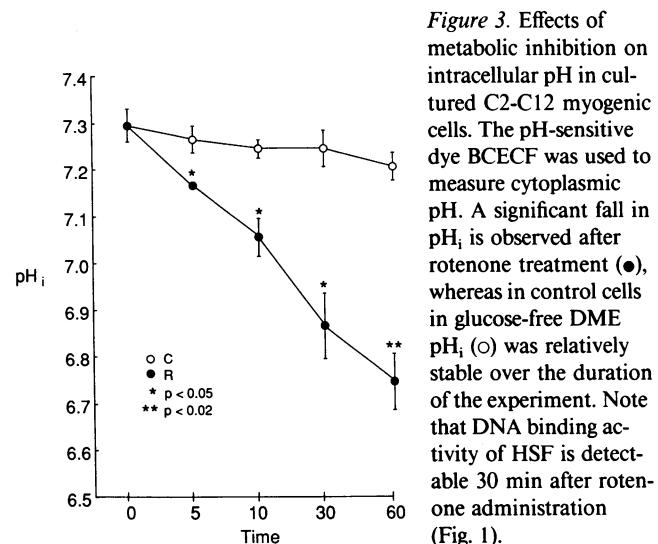


**Figure 2.** Competition experiments in gel mobility shift assays were performed with whole-cell extracts from C2-C12 myogenic cells after either heat-shock (lanes 1–5) for 15 min at 42°C or rotenone treatment (lanes 6–9) for 30 min. Complete competition was observed in the presence of 200-fold molar excess of cold HSE but not with equivalent molar amounts of a noncomplementary oligonucleotide, thus confirming that the specificity of HSF-DNA protein interactions during heat shock and metabolic inhibition was similar.

tions, which fell to 30% of control values after 30 min, a time point corresponding to the onset of HSF-DNA binding activity (Figs. 1 and 4). During glucose deprivation, ATP fell to 50% of control levels within 1 h with only minimal drop in  $\text{pH}_i$ . Under these conditions HSF-DNA binding activity was not observed.

#### Dissociation of DNA binding activity from cellular acidosis.

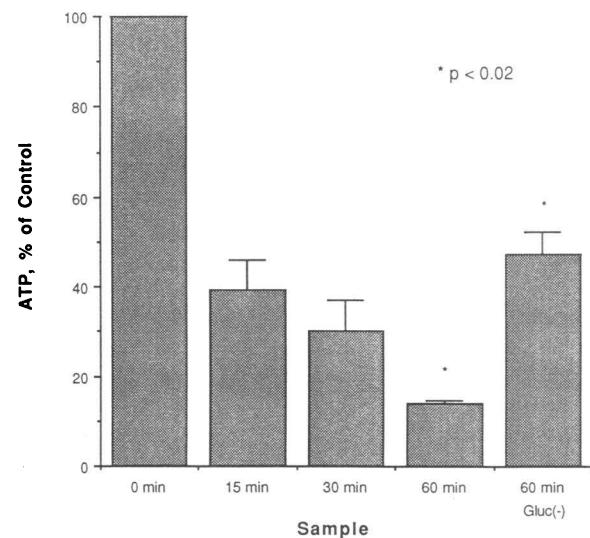
In an effort to dissociate the effects of ATP depletion and acidosis, C2C12 myogenic cells were exposed to propionic acid and amiloride and the time course of change in  $\text{pH}_i$  and DNA-binding activity of HSF were compared. Propionic acid ( $4 \times 10^{-3}$  M) acidifies cells by nonionic diffusion of the acid into the cell; amiloride ( $1 \times 10^{-3}$  M) was added to inhibit the  $\text{Na}/\text{H}$  antiporter and allow the cellular acidosis to persist. In the absence of amiloride, cellular acidosis was transient and  $\text{pH}_i$  returned to control levels within 1 min. Interestingly, despite severe acidosis in cells treated with propionic acid and amiloride ( $\text{pH}_i < 6.7$ ) that was achieved within 5 min and sustained for 1 h, DNA binding activity of HSF was not detected (data not shown).



**Figure 3.** Effects of metabolic inhibition on intracellular pH in cultured C2-C12 myogenic cells. The pH-sensitive dye BCECF was used to measure cytoplasmic pH. A significant fall in  $\text{pH}_i$  is observed after rotenone treatment (●), whereas in control cells in glucose-free DME  $\text{pH}_i$  (○) was relatively stable over the duration of the experiment. Note that DNA binding activity of HSF is detectable 30 min after rotenone administration (Fig. 1).

## Discussion

Our results support several conclusions concerning the changes in the intracellular milieu of ischemic cells that are associated



**Figure 4.** Effects of metabolic inhibitor, rotenone ( $2 \times 10^{-4}$  M), on ATP pools. C2C12 myogenic cells were incubated with rotenone or in glucose-free DME lacking the inhibitor for the indicated times under normoxic conditions at 37°C. After extraction in 60% methanol, ATP levels were determined by HPLC. ATP pools fell to 30% of control after 30 min when DNA binding of HSF was detectable. Data shown are from three separate experiments performed in either duplicate or triplicate.

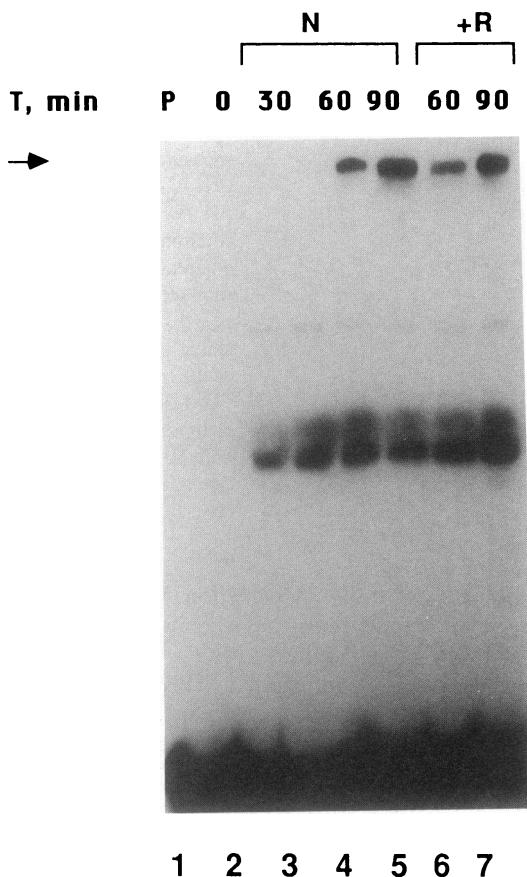


Figure 5. Effects of ATP depletion on HSF-DNA binding activity at physiological pH. To maintain normal pH at 7.4, the cells were incubated at the indicated times in glucose-free DME (pH 7.4) supplemented with high K<sup>+</sup> (120 meq)/nigericin without (N) or with rotenone (+R). Both treatments induce the activation of HSF-binding activity, see lanes 4–7 and deplete high-energy phosphates pools (data not shown).

with transcriptional activation of stress protein genes. First, because the effects of hypoxia are reproduced by metabolic inhibition under normoxic conditions, hypoxic activation of HSF is unlikely to be attributable to a direct oxygen-sensing mechanism (reference 12 and this study). Second, moderate levels of ATP depletion (< 50% of control levels), as seen with glucose deprivation, do not induce the DNA-binding activity of HSF, at least when pH<sub>i</sub> remains normal. Third, severe acidosis is not a sufficient stimulus for HSF activation when ATP stores are preserved. Finally, although the combined effects of depletion of high-energy phosphate stores (ATP < 30% of control) coupled with a fall in pH<sub>i</sub> lead to the activation of HSF, ATP depletion alone is a sufficient stimulus to achieve this response.

Recently Mosser et al. (14) reported that HSF-DNA binding in vitro could be induced by reduction of pH to nonphysiologic levels (< 6.2). They attributed this activation to conformational changes in proteins induced by the fall in pH. The results of our present study indicate that even severe acidosis (pH<sub>i</sub> 6.7) in the physiological range does not induce HSF-binding activity in intact cells. Moreover, our findings are consistent with studies in *Drosophila* in which maneuvers that lowered pH<sub>i</sub> to 6.8 failed to activate the characteristic puffs of polytene chromosomes seen after heat shock (19). Although

our results would not exclude a synergistic or additive effect of acidosis during ischemic conditions, the principal stimulus for HSF activation appears to be distinct from the decline in pH<sub>i</sub>.

A common feature of stresses such as heat, amino acid analogues, and metabolic inhibition appears to be the production of unfolded or abnormal proteins (20). There is considerable evidence to implicate the direct participation of both constitutive and inducible members of the major HSP70 multigene family in assembly, transport, and refolding of nascent or denatured proteins (21, 22). In addition, several investigators have demonstrated the important role of stress proteins in energy-dependent cellular processes such as protein translocation into cellular organelles (22–24). Moreover, studies that show HSP70s to possess ATPase activity and the three-dimensional structure of bovine constitutive HSP70 to contain the nucleotide binding-pocket in the 44-kD amino-terminal fragment (25) support the general scheme in which the interaction of stress proteins and their target substrate polypeptides requires ATP-hydrolysis (21, 23, 25–27).

What are the molecular mechanisms by which HSF may be activated by ATP depletion? We and others propose that the DNA binding domain of HSF is masked in unstressed cells by complex formation with HSP70 (11, 14). As a consequence of ATP depletion, HSP70 proteins complexed to nascent or unfolded protein cannot be recycled, thereby reducing the free pool of HSP70 available to complex with HSF (22). In addition, ATP depletion also may augment the intracellular load of denatured and/or unfolded proteins, thereby increasing the demand for HSP70 and further depleting the pool of free HSP70. Finally, if the affinity of HSP70 for HSF is lower than its affinity for unfolded proteins, the depletion of free HSP70 will release HSF for nuclear translocation, DNA binding, and transcriptional activation (28, 29). Although some aspects of our proposal are speculative, this reconciles our findings with current concepts of activation of HSF during thermal stress. Moreover, several features of this hypothesis are experimentally testable in further investigations.

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