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NOTES

Regulation and Mechanism of Action of the Small Heat Shock Protein from the Hyperthermophilic Archaeon *Pyrococcus furiosus*†

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The small heat shock protein (sHSP) from the hyperthermophile *Pyrococcus furiosus* was specifically induced at the level of transcription by heat shock at 105°C. The gene encoding this protein was cloned and overexpressed in *Escherichia coli*. The recombinant sHSP prevented the majority of *E. coli* proteins from aggregating in vitro for up to 40 min at 105°C. The sHSP also prevented bovine glutamate dehydrogenase from aggregating at 56°C. Survivability of *E. coli* overexpressing the sHSP was enhanced approximately sixfold during exposure to 50°C for 2 h compared with the control culture, which did not express the sHSP. Apparently, the sHSP confers a survival advantage on mesophilic bacteria by preventing protein aggregation at supraoptimal temperatures.

Hyperthermophilic microorganisms that can grow at or above 100°C are now fairly well known. However, little is known about their adaptive responses to extremely high temperatures. Ubiquitous heat shock responses are observed when organisms are confronted by near-lethal temperatures. Sets of proteins with diverse functions known as heat shock proteins (HSPs) are induced in response to abrupt temperature changes (11). The synthesis of these proteins can lead to acquired thermotolerance, allowing the organisms to survive at even higher temperatures (9, 21, 33, 36). HSPs have been divided into five classes based on their molecular weights: HSP100, HSP90, HSP70, HSP60, and small HSPs (sHSPs) (35). Most HSPs function as molecular chaperones, catalyzing refolding of denatured proteins, assisting maturation of newly synthesized proteins, or suppressing protein aggregation (7, 8, 10, 22).

sHSPs are a common class of HSPs; their molecular masses range from 15 to 42 kDa, and they normally form multisubunit complexes of 200 to 800 kDa (13). Some sHSPs are abundant in nonstressed cells (18), but others are stress induced (20, 29). Most sHSPs share a conserved motif with α-crystallin proteins, which are ubiquitous proteins found in vertebrate eye lenses and which are known to function as molecular chaperones (10, 15, 34). The in vivo functions of sHSPs remain unclear. It has been proposed that they confer thermotolerance or tolerance to chemical challenges such as superoxide (17). Survival of an HSP30-deficient mutant of *Neurospora crassa* under high-temperature, carbohydrate-limited conditions was shown to be reduced dramatically compared to survival of the wild type (26). However, mechanisms of action have not been defined.

In particular, the functions and regulation of archaeal sHSPs remain unclear, although the sHSP from the hyperthermophilic methanogenic archaeon *Methanococcus jannaschii* has been cloned and expressed and its crystal structure has been reported (14). Based on its crystal structure, the *M. jannaschii* sHSP forms oligomeric structures having 24 subunits of the 16.5-kDa monomer. We have studied the sHSP from *Pyrococcus furiosus* (Pfu-sHSP), a hyperthermophilic archaeon that grows optimally at about 100°C (4). We report the cloning and regulation of Pfu-sHSP, the effects of its overexpression in *Escherichia coli*, and the mechanism of its action in stabilizing a mesophilic protein at elevated temperatures.

Sequence comparison and phylogenetic analysis. The Pfu-sHSP gene was identified from the genome sequence (27) based on its similarity to other α-crystallin-like sHSP genes. The protein is composed of 167 amino acids encoded by an open reading frame of 504 nucleotides (GenBank accession no. AF256212) and has a predicted pI of 5.25. The low-molecular-weight sHSPs have significantly higher diversities in size and amino acid sequence than do the higher-molecular-weight HSPs (24). The amino acid sequence of Pfu-sHSP showed low similarity to the sHSP of *Clostridium acetobutylicum* and to other bacterial and eukaryotic sHSPs (31). Typically, a nonconserved region occurs at the amino terminus (14).

Comparison of the amino acid sequences of Pfu-sHSP with other sHSPs by BestFit (Wisconsin Package, version 10.0; Genetics Computer Group, Madison, Wis.) reveals, not surprisingly, that the sHSP of the marine hyperthermophilic archaeon *Pyrococcus horikoshii* (5) has the highest percent identity and similarity to Pfu-sHSP. Interestingly, the sHSP from the bacterium *Aquifex aeolicus* is more similar to that of *P. furiosus* than are sHSPs from archaea with lower optimal growth temperatures. The sHSP from the mesophilic bacterium *C. acetobutylicum* shows the lowest percent identity to Pfu-sHSP (Table 1).

Native Pfu-sHSP and its mRNA are induced by heat shock. In order to determine whether Pfu-sHSP is regulated in re-

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TABLE 1. Amino acid similarity of Pfu-sHSP to corresponding proteins of other organisms

Organism	Comparison to Pfu-sHSP[a]		Reference
	% Identity	% Similarity	
Pyrococcus horikoshii	87.3	91.6	12
Aquifex aeolicus	43.0	59.8	2
Archaeoglobus fulgidus	39.2	51.5	19
Methanobacterium thermoautotrophicum	37.3	52.7	33
Oryza sativa	34.0	47.9	38
Methanococcus jannaschii	33.8	40.1	14
Clostridium acetobutylicum	31.0	45.0	31

[a] Percent identity and percent similarity were calculated from an alignment of the Pfu-sHSP amino acid sequence with other sHSPs by BestFit (Genetics Computer Group, Madison, Wis.).

sponse to heat shock, we obtained polyclonal antiserum against Pfu-sHSP by immunization of a rabbit with purified, recombinant Pfu-sHSP (BioWorld, Dublin, Ohio). Western blot analysis using this antiserum demonstrated that Pfu-sHSP is indeed heat shock inducible. *P. furiosus* was cultured in a modified 20-liter fermentor (New Brunswick) under standard conditions with 5 g of S^0/liter (1). The cultures were incubated at 95°C for 4 h and then shifted to 105°C for 0, 30, 60, or 120 min before being chilled on ice and harvested by centrifugation at 7,500 × *g* for 15 min. Heat shock was carried out at 105°C because the maximum temperature for growth of *P. furiosus* is 103°C (4). The total protein levels of the cell extracts were measured, and equal amounts of protein were loaded onto each lane of a sodium dodecyl sulfate (SDS)-polyacrylamide gel (Fig. 1A). Western blot analysis was done as described elsewhere (30). A strong signal was observed at 20 kDa (Fig. 1B, lanes 2 to 4), corresponding to 30, 60, and 120 min after the onset of heat shock, whereas no signal was observed for the non-heat-shocked control culture (Fig. 1B, lane 1). This indicates that native Pfu-sHSP is strictly heat inducible and that Pfu-sHSP is apparently not required for rapid growth at the optimal growth temperature.

We measured the expression of mRNA from the gene encoding Pfu-sHSP under heat shock conditions by Northern blot analysis (30). Total RNA was isolated from *P. furiosus* after exposure to 105°C for 120 min and compared to a non-heat-shocked control culture. Total RNA (4 μg) was electrophoresed on a 1.5% agarose gel for Northern blot analysis with a radiolabeled PCR probe from the Pfu-sHSP gene generated by PCR amplification using [^{32}P]dCTP. Hybridization with this probe revealed a transcript of 600 nucleotides, corresponding to the size of the putative Pfu-sHSP gene (Fig. 1C). A radiolabeled probe from the gene encoding *P. furiosus* glutamate dehydrogenase (GDH), which is expressed constitutively (3, 32), was used as a control (Fig. 1D).

Cloning, overexpression, and purification of Pfu-sHSP in *E. coli*. The region encoding the 504-nucleotide Pfu-sHSP gene was amplified from *P. furiosus* genomic DNA by PCR using primers Pfu-shspN, containing an *Nco*I site (C′CATG_G [underlined in the full primer sequence]) (5′GCCATGGTGAGG AGAATAAGAAGATGG), and Pfu-shspC, containing an *Xho*I site (C′TCGA_G [underlined in the full primer sequence]) (5′ACTCGAGCTATTCAACTTTAACTTCGAAT CCTTC). The gene fragment was cloned into the pCR Zero Blunt vector (Invitrogen, Carlsbad, Calif.). The insert was digested by *Nco*I and *Xho*I and then subcloned into the IPTG (isopropyl-1-thio-β-D-galactopyranoside)-inducible pET19b expression vector (Novagen, Madison, Wis.). The construct was

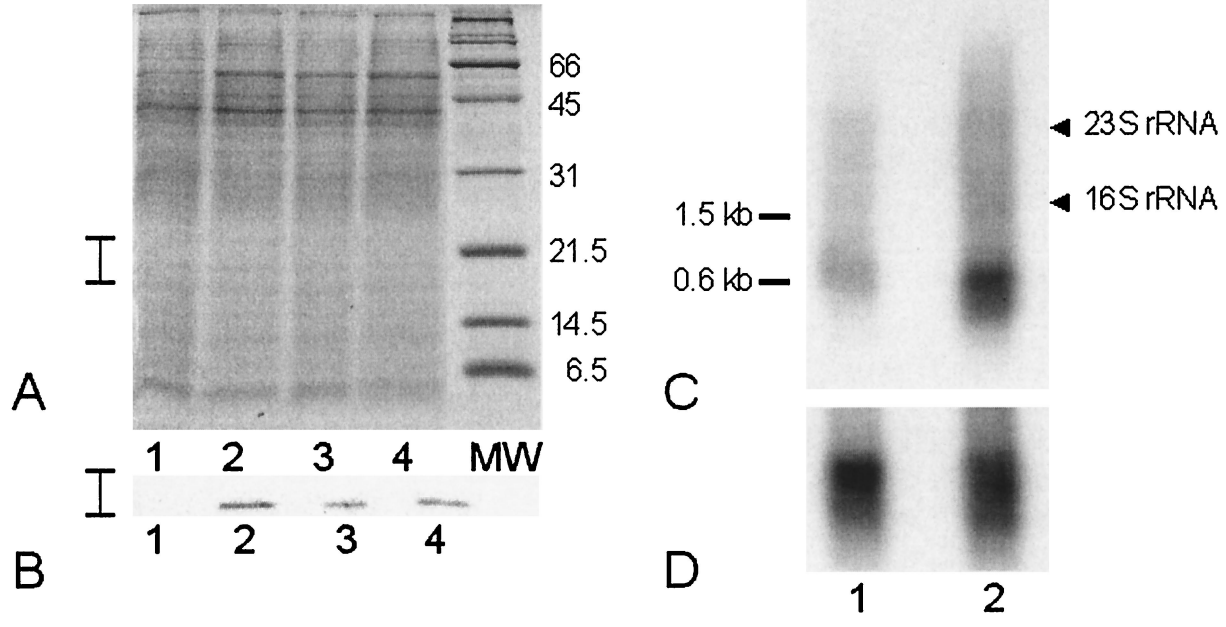

FIG. 1. Expression of native Pfu-sHSP. Protein expression is shown by SDS–15% PAGE and Western blot analysis in panels A and B, respectively. (A and B) Lanes 1, culture growing at 95°C; lanes 2, 3, and 4, *P. furiosus* cultures after heat shock treatment at 105°C for 30, 60, and 120 min, respectively. The vertical bars on the left show the section of the gel represented in the Western blot analysis. Northern blot analyses of Pfu-sHSP gene mRNA expression and *P. furiosus* GDH gene mRNA (control) expression are shown in panels C and D, respectively. (C and D) Lanes 1, autoradiogram of a blot of mRNA from non-heat-shocked cells; lanes 2, corresponding mRNA signals from heat-shocked cells.

designated pPfu-shsp. *E. coli* BL21(DE3) (Novagen), carrying pSJS1240, which encodes the rare *E. coli* Arg-tRNA^{AGA} and Ile-tRNA^{ATA} (16), was used as an expression host. *E. coli* cultures were grown in Luria-Bertani broth in the presence of 50 μg each of ampicillin and spectinomycin per ml to an A_{595} of 0.6. Pfu-sHSP expression was induced by the addition of 1 mM IPTG for 3 h. The same strain carrying pET19b and pSJS1240 was the negative control. SDS-polyacrylamide gel electrophoresis (PAGE) of *E. coli* overexpressing Pfu-sHSP crude extract revealed an additional protein of 20 kDa, which corresponds to the protein molecular weight deduced from the predicted amino acid sequence. After induction, *E. coli* cells overexpressing Pfu-sHSP were harvested and resuspended in 25 mM potassium phosphate buffer (pH 7.0)–2 mM dithiothreitol–1 mM EDTA (buffer A). The cells were disrupted using a French press (SLM Instruments, Urbana, Ill.) at 16,000 lb/in^2, and the extract was centrifuged at 5,000 × *g* for 15 min. Pfu-sHSP appeared in the particulate fraction as indicated by SDS-PAGE. The pellet was washed and dissolved in buffer A by heating at 85°C for 20 min. The dissolved pellet was then filtered and loaded onto an anion-exchange column (MonoQ; Pharmacia Biotech, Uppsala, Sweden) previously equilibrated with buffer A. Pfu-sHSP was eluted as a single peak at 0.35 M NaCl by using a linear gradient from 0 to 1 M NaCl. Fractions were subsequently pooled and concentrated.

Protection of *E. coli* cell extracts by Pfu-sHSP at 105°C. Several sHSPs are known to prevent aggregation of proteins during heating in vitro (15, 23). We examined the effect of Pfu-sHSP expressed in *E. coli*. The cells were harvested and prepared as described above. The total protein concentration was determined using the Bradford protein assay kit (Bio-Rad, Hercules, Calif.). The cell extracts were diluted in buffer A to a uniform protein concentration of 4 mg/ml. Diluted cell extracts were covered with mineral oil and heated at 105°C for 0, 20, 30, and 40 min in 1.5-ml microcentrifuge tubes. After being cooled to room temperature, the samples were centrifuged at 10,000 × *g* for 5 min at 25°C and the supernatants were collected. The residual proteins were visualized by SDS-PAGE and subjected to the Bradford protein concentration assay. At least 90% of the proteins in the *E. coli* cell extracts containing overexpressed Pfu-sHSP remained soluble after heat treatment for 40 min and appeared in the supernatant fractions (Fig. 2). Approximately 30% of the soluble proteins remained in the control supernatants after heat treatment. SDS-PAGE showed that high-molecular-weight proteins of *E. coli* were protected by Pfu-sHSP, whereas those in the control were rapidly aggregated at 105°C (Fig. 2). This finding indicates that sHSP prevents aggregation of many different proteins at or above their denaturing temperatures.

Pfu-sHSP prevents aggregation of mesophilic GDH as a result of heat inactivation. Since Pfu-sHSP can prevent aggregation of other proteins in response to heat stress, we addressed the question of whether or not it could extend the half-life ($t_{1/2}$) of a purified enzyme in vitro. Bovine glutamate dehydrogenase (boGDH) (Sigma, Milwaukee, Wis.) was used as a model. boGDH is a mesophilic enzyme with an optimal assay temperature of 25°C that is inactivated rapidly at 56°C. Purified Pfu-sHSP was added to solutions of boGDH during heat treatment at 56°C in EPPS (*N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid) buffer, pH 8.0, to a boGDH concentration of 0.9 mg/ml, with 2.25 mg of purified Pfu-sHSP/ml. Samples were removed and assayed at 0, 2, 4, and 8 min and centrifuged at 10,000 × *g* for 2 min. The residual activities of boGDH in the supernatant samples were assayed as described previously (28) using a Beckman DU640 spectrophotometer with the temperature controlled at the optimum, 25°C. The assay mixture contained 100 mM EPPS (pH 8.0), 65 mM glutamic acid, and 16.25 mM NADP. There was no detectable GDH activity in the purified Pfu-sHSP (data not shown).

The $t_{1/2}$ for boGDH precipitation was measured by centrifugation and recording of the A_{280} of the supernatant. In the experiment in which boGDH was incubated alone, the apparent $t_{1/2}$ was approximately 2 min, whereas the boGDH to which

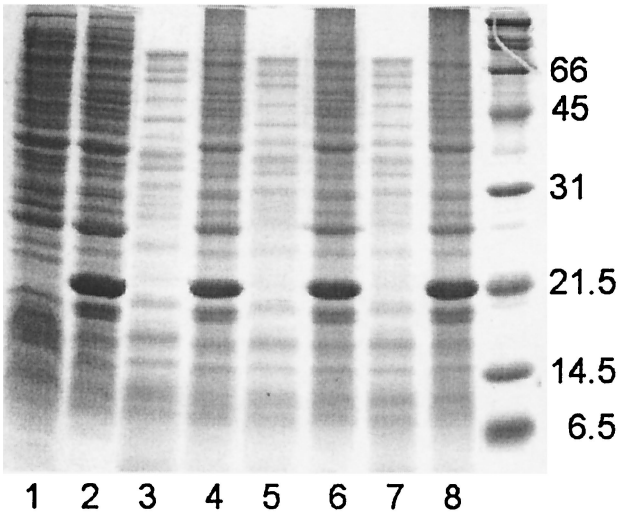

FIG. 2. SDS–15% PAGE analysis of thermal protection of *E. coli* crude extracts by Pfu-sHSP at 105°C. Lanes 1 (*E. coli* crude extracts without Pfu-sHSP) and 2 (*E. coli* with overexpressed Pfu-sHSP) represent control experiments without heat treatment. Lanes 3, 5, and 7 show *E. coli* crude extracts without overexpressed Pfu-sHSP heated to 105°C for 20, 30, and 40 min, respectively; lanes 4, 6, and 8 show *E. coli* crude extracts with overexpressed Pfu-sHSP heated to 105°C for 20, 30, and 40 min, respectively.

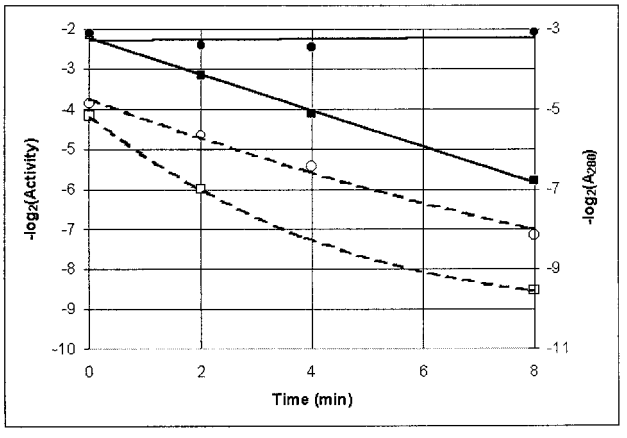

FIG. 3. Effects of Pfu-sHSP on boGDH incubated at 56°C for up to 8 min. Solubility is shown as $\log_2 A_{280}$ of boGDH in the presence (●) and absence (■) of Pfu-sHSP (solid lines). Activity of boGDH is shown in the presence (○) and absence (□) of Pfu-sHSP (dashed lines).

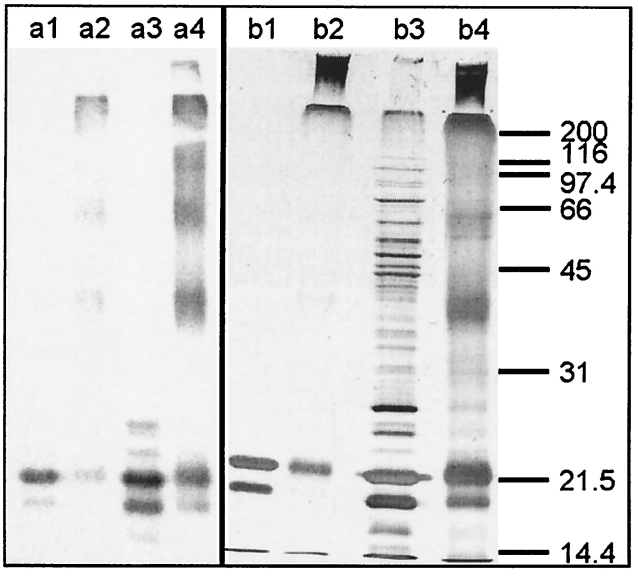

FIG. 4. Cross-linking of purified Pfu-sHSP and Pfu-sHSP in unpurified *E. coli* extracts as visualized by Western blot analysis (a) and silver-stained SDS–12% PAGE (b). Lanes 1, un-cross-linked purified Pfu-sHSP; lanes 2, cross-linked purified Pfu-sHSP; lanes 3, un-cross-linked *E. coli* extracts; lanes 4, cross-linked *E. coli* extracts.

sHSP was added did not precipitate at all during the course of the experiment. The activity of boGDH in the supernatants, on the other hand, declined in both cases (Fig. 3). Thus, much of the boGDH that remained in solution was soluble but inactive. In this case, the enzyme was maintained in solution but not preserved from denaturation. This is an important result, indicating that the probable mode of action of Pfu-sHSP is toward aggregation of nonnative proteins, thus allowing them to be recruited to either refolding or protein turnover pathways.

We addressed the question of the subunit structure of Pfu-sHSP by incubating purified Pfu-sHSP and *E. coli* extracts containing expressed Pfu-sHSP in 0.01% (wt/vol) glutaraldehyde to cross-link the proteins. The control and cross-linked preparations were subjected to SDS-PAGE and visualized by silver staining and Western blotting (Fig. 4). In contrast to *M. jannaschii* sHSP, which is reported to occur mostly as 24-mers (14), Pfu-sHSP appears to be polydisperse. This characteristic has been reported for several other α-crystallin homologs (6). The polydisperse patterns of the Pfu-sHSP polymer from purified Pfu-sHSP and from *E. coli* crude extracts are indistinguishable.

Survivability at 50°C of *E. coli* overexpressing Pfu-sHSP. sHSPs from the chestnut (*Castanea sativa*) and from murine and human αβ-crystallin have been shown to confer slight thermotolerance at 50°C on *E. coli* (23, 25, 34). We show here that an archaeal sHSP can also confer very significant thermotolerance on a typical mesophilic bacterium. *E. coli* cultures containing pPfu-shsp/pSJS1240 were induced as described above, and the cells were diluted in Luria-Bertani broth containing 50 μg each of ampicillin and spectinomycin per ml to an A_{595} of 0.6. The cultures were rapidly shifted to 50°C in a water bath shaker. Samples were removed at 0, 20, 40, 60, and 120 min, diluted appropriately, and plated on Luria-Bertani agar containing 50 μg each of ampicillin and spectinomycin per ml, and the plates were incubated at 37°C overnight. Cell viability was determined by counting of CFU after overnight incubation. The first-order rate of decline of viability of *E. coli* overexpressing Pfu-sHSP was significantly higher, approximately six- to sevenfold, than that of the culture transformed with pET19b and pSJS1240 (Fig. 5). As a result, the difference in viability between protected and unprotected cells after a 120-min exposure at 50°C was approximately 50-fold.

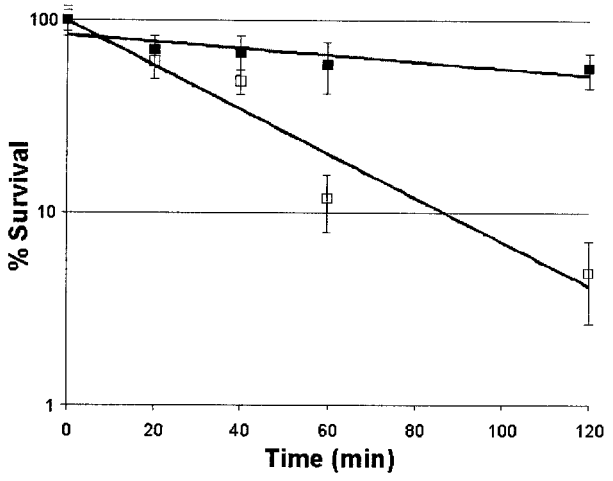

FIG. 5. Effect of recombinant Pfu-sHSP expression on *E. coli* viability at 50°C. The reduction of viability as an exponential function of time is shown. □, pET19b/pSJS1240; ■, pPfu-shsp/pSJS1240.

We conclude that Pfu-sHSP increases the thermotolerance of *E. coli* by preventing aggregation of heat-compromised proteins. Because many proteins from hyperthermophiles are nonfunctional at the optimal growth temperatures of mesophiles, it is intriguing that a component of the adaptive response of an archaeon growing at 100°C can enhance the heat resistance in *E. coli* cells growing at "mesophilic" temperatures. However, Trent et al. found that the chaperonin TF55 from *Sulfolobus shibatae* can bind a denatured protein at 25°C as efficiently as at 70°C (37). These findings will enable us to assess the biological activity of sHSP mutants by using *E. coli* as a surrogate genetic system for *P. furiosus*, which lacks systems for mutation selection and recombinant gene expression.

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