Induction of a heat shock-like response by unfolded protein in *Escherichia coli*: dependence on protein level not protein degradation

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To test the idea that unfolded protein might act as an intracellular signal for induction of the heat shock response in *Escherichia coli*, we examined the synthesis of several heat shock proteins after expression of an unfolded variant of the amino-terminal domain of λ repressor. These experiments show that expression of a single mutant protein, and not its wild-type counterpart, is sufficient to induce a heat shock-like response. In addition, by measuring the abilities of unfolded variants of differing proteolytic susceptibilities to induce heat shock protein synthesis and by monitoring heat shock protein synthesis as a function of the amount of a single unfolded protein, we show that it is the concentration of unfolded protein in the cell, and not its degradation, that is important for inducing the heat shock-like response.

*Key Words:* Heat shock response; unfolded protein; protein degradation; *Escherichia coli*; λ repressor

Received February 23, 1989; revised version accepted May 23, 1989.

*Escherichia coli*, like most organisms, responds to an increase in temperature by increasing the synthesis of a set of proteins called heat shock proteins (HSPs; for review, see Neidhardt et al. 1984). Treatment of cells with other stressors such as ethanol and hydrogen peroxide also induces some or all of the heat shock proteins (VanBogelen et al. 1987).

A key regulator of the heat shock response is σ^32, the product of the htpR gene, which functions as an RNA polymerase subunit to direct transcription from specific heat shock promoters (Grossman et al. 1984). After heat shock, the intracellular level of σ^32 increases, resulting in elevated transcription of HSP genes (Straus et al. 1987). At present, it is not certain how heat shock and other stress treatments are coupled to increased transcription by σ^32. Because many of the inducing treatments can lead to protein damage or denaturation, it has been suggested (Goff and Goldberg 1985) that production of unfolded polypeptides may be a universal signal for induction of the heat shock response. In fact, HSP induction occurs after treatment of *E. coli* cells with amino acid analogs and puromycin, which are thought to increase the intracellular concentration of unfolded protein, and after expression of certain ‘foreign’ proteins whose rapid degradation is taken as evidence for an unfolded structure (Goff and Goldberg 1985).

In the experiments reported here, we test directly the idea that unfolded protein can act as a signal for induction of heat shock proteins. Using the amino-terminal domain of λ repressor, a protein of known structure (Pabo and Lewis 1982), we show that expression of an unfolded, mutant polypeptide and not its folded, wild-type counterpart is sufficient to induce a heat shock-like response in *E. coli*. Moreover, we show that HSP induction does not depend on the degradation of unfolded protein, but rather, on its intracellular concentration. This suggests a direct role for the unfolded protein in the induction process.

**Results**

*Folded and unfolded variants of the amino-terminal domain*

To test the idea that expression of unfolded protein is sufficient to induce a heat shock-like response, we use two variants of the amino-terminal domain of λ repressor. The wild-type fragment is designated N_{102LT}. The amino-terminal 92 residues of this fragment [128 residues total] fold into a stable, predominantly α-helical, three-dimensional structure, whose stability can be monitored by changes in circular dichroism. As shown in the thermal denaturation experiment of Figure 1, the α-helical portion of the wild-type N_{102LT} protein is...
folded stably at 37°C and unfolds with a $T_m$ near 55°C. A mutant form of this fragment, designated LA57-N°2LT, contains a single amino acid substitution (Leu°7 → Ala), which reduces its $T_m$ to ~25°C [Fig. 1]. At 37°C the α-helical portion of this mutant protein is unfolded in ~80% of the molecules. Because the amino-terminal domain of λ repressor contains no disulfide bonds and because the experiments shown in Figure 1 were conducted at roughly physiological pH and ionic strength, the estimate of the fraction of protein in the unfolded form should be a reasonable approximation of that found in vivo (Parsell and Sauer 1989).

Expression of unfolded protein induces a heat shock-like response

In separate experiments, tac promoter-mediated expression of the wild-type and mutant forms of the N°2LT protein was induced by addition of isopropyl-thio-β-D-galactoside (IPTG), and synthesis of E. coli proteins was monitored by 2-min [35S]methionine pulses at intervals thereafter. Expression of the thermally unstable mutant results in increases in the synthesis rates of several heat shock proteins including GroEL, DnaK, and C62.5, as shown in Figure 2A. Increased synthesis of a lacZ fusion protein from the $p_{lac}$ heat shock promoter also is induced by expression of the unfolded protein. No significant increase in the synthesis of heat shock proteins is observed when expression of the folded N°2LT protein is induced with IPTG [Fig. 2B]. This latter result indicates that the increased synthesis of heat shock proteins in the former case is not caused directly by the addition of IPTG or indirectly via expression of proteins other than LA57-N°2LT whose synthesis might be under IPTG control. Thus, the expression of a single, predominantly unfolded protein is sufficient to induce a heat shock-like response.

As shown in Figure 2A, increased heat shock protein synthesis is evident 10 min after IPTG addition, with synthesis rates increasing somewhat at 20 and 30 min. In experiments not shown, we observed that HSP synthesis reached a plateau at 25–40 min after addition of IPTG and then remained constant until 60 min [the longest time tested]. The experiment shown in Figure 2 was performed at 37°C. Experiments performed at 30°C gave similar results. Also, we found that HSP induction did not depend on the particular destabilizing mutant substitution [Leu°7 → Ala] used for these studies. Other destabilizing substitutions at position 57 (e.g., Gly, Cys, Pro, and Arg) or at other hydrophobic core positions (e.g., Leu°8 → Arg or Glu) also resulted in proteins whose expression induced increased HSP synthesis [data not shown].

Neither overproduction of the plasmid-borne repressor proteins nor the resulting induction of heat shock proteins in the case of the unfolded mutant appears to be deleterious to the cell. As shown in Figure 3, no significant changes in growth rate can be detected for several generations after IPTG induction of the wild-type or mutant amino-terminal domain proteins. We also examined the induction of HSPs after the expression of unfolded protein in isogenic lon° and lon° strains and htpR° strains and htpR° strains. Although deletion of the lon gene had no effect on the ability of the LA57-N°2LT protein to induce increased HSP synthesis, an amber mutation in the htpR gene [suppressed by a temperature-sensitive suppressor at 30°C] prevented this induction [data not shown]. Although some σ°2 may be present in the htpR° strain at 30°C, the absence of induction of HSPs after expression of the LA57-N°2LT protein suggests that σ°2 or a protein under σ°2 control is required at some step for the heat shock-like response. On the other hand, the product of the lon gene, protease La, clearly is not required for the ability of unfolded LA57-N°2LT to induce HSP synthesis.

HSP induction does not depend on the rapid degradation of unfolded protein

Is HSP induction dependent on the concentration of unfolded protein or on the degradation of this protein? To address this issue, we used the amino-terminal domain variants LA57-N°2LT and LA57-N°3. Both of these proteins contain the same 92 amino-terminal amino acids, which are predominately unfolded at 37°C because each contains the LA57 mutation ([Fig. 1; Parsell and Sauer 1989]. However, as shown in the pulse-chase experiments of Figure 4, the proteins have different intracellular half-lives. The LA57-N°2LT protein is quite stable with a half-life of >3 hr, whereas the LA57-N°3 protein is degraded rapidly with a half-life of ~6 min. We have shown previously that the different intracellular stabilities of these proteins arise because they have different carboxy-terminal sequences [Bowie and Sauer 1989; Parsell and Sauer 1989]. Compared with the N°3 protein, the N°2LT protein has a 38-residue carboxy-terminal extension.
Expression of the two LA57 amino-terminal-domain variants induces HSP synthesis to differing extents. As shown in Figure 5, expression of the LA57-N102LT protein induces HSP synthesis more efficiently than does expression of the LA57-N92 protein. Thus, there is an inverse correlation between degradation rates and the ability to induce HSP synthesis. The protein that has the longer intracellular half-life is the most effective at inducing HSP synthesis, whereas the protein that has the shorter half-life is the least effective. Both of the amino-terminal domain proteins are synthesized at approximately the same rate (data not shown); therefore, it seems likely that the LA57-N102LT protein induces HSP synthesis more efficiently that the LA57-N92 variant simply because it is present at a higher level. Thus, HSP synthesis in E. coli appears to depend on the intracellular concentration of unfolded protein and not on the rapid degradation of that protein.

In principle, the different abilities of the LA57 variants to induce heat shock protein synthesis could be directly related to their different carboxy-terminal extensions. However, the carboxy-terminal residues of the LA57-N102LT protein also are present in the N102LT protein, which does not induce heat shock protein synthesis (Fig. 2), even though it is present at a high steady-state level (data not shown). In the next section we test directly the idea that the induction of HSPs depends on the concentration of unfolded protein.

Figure 2. Synthesis of heat shock proteins after expression of unfolded and folded proteins. At time zero [0], 1 mM IPTG was added to logarithmic-phase cells of strain DP700 (grown at 37°C) bearing plasmids encoding either the predominantly unfolded [LA57-N102LT] or folded [N102LT] protein, and aliquots were pulse-labeled for 2 min at intervals thereafter. [Lane HS] An uninduced culture of LA57-N102LT pulse-labeled 7 min after a temperature shift from 37°C to 42°C. Induced heat shock proteins are indicated by arrows with the uppermost arrow showing the position of the fusion protein product made from the P\textit{prp}G promoter.

Figure 3. Expression of wild-type or mutant amino-terminal domain variants does not affect the growth of E. coli strain DP700. At OD\textsubscript{600} = 0.2–0.3, logarithmic-phase cultures were split and expression of plasmid-encoded protein was induced with 1 mM IPTG in half of the culture. □ Uninduced cells; ○ induced cultures.
Unfolded protein and heat shock

Figure 4. Degradation of LA57-N102LT and LA57-N92 in E. coli strain DP700. The fraction of pulse-labeled protein remaining at various times after a chase was determined by densitometry of autoradiographs.

Induction of HSPs is directly correlated with the intracellular level of unfolded protein

The dependence of HSP synthesis on the concentration of unfolded protein also can be seen when the level of a single protein is varied. In the experiments of Figure 6, tac promoter-mediated expression of the LA57-N102LT protein was adjusted by varying the amount of IPTG present during a 30-min induction period. The cells then were pulse-labeled with [35S]methionine and samples were electrophoresed and either autoradiographed to monitor protein synthesis [Fig. 6A] or Western-blotted to monitor accumulation of LA57-N102LT protein [Fig. 6B]. Over the range of IPTG concentration shown, increased synthesis of heat shock proteins can be seen to occur concomitantly with increased levels of LA57-N102LT protein. We believe that these data and those shown in Figure 5 provide strong support for the idea that the level of unfolded protein present in the cell, and not its degradation, determines its ability to act as an inducer of heat shock protein synthesis.

Discussion

We have shown that the expression of a single unfolded polypeptide is sufficient to induce increased synthesis of heat shock proteins in E. coli. These experiments extend the work of other investigators [Goff and Goldberg 1985, Ito et al. 1986], which showed that expression of 'abnormal' polypeptides could induce heat shock protein synthesis. In addition, using variants of the amino-terminal domain of λ repressor, we have shown that the ability of a given unfolded protein to induce HSP synthesis correlates with its intracellular concentration rather than its degradation rate. At present, we do not know whether all unfolded chains are equivalent in their ability to induce HSPs or whether there are specific sequence or structural elements that, in the context of an unfolded polypeptide, are recognized as signals for HSP induction.

Is an increase in the concentration of unfolded protein the normal physiological signal for induction of the heat shock response? This model is attractive for several reasons: (1) Increased levels of some unfolded proteins can induce a heat shock-like response; (2) many of the environmental treatments that induce heat shock could result in protein denaturation; and (3) recent studies suggest that some heat shock proteins may function by binding to unfolded polypeptides [Bochkareva et al. 1988]. If heat shock proteins function, at least in part, to protect the cell from the deleterious effects of unfolded proteins, it would be appropriate that an increase in the concentration of unfolded protein could act as a signal for induction of the heat shock response.

The kinetics of HSP induction normally observed after a temperature increase differ from those observed here after expression of unfolded protein. After an increase in temperature, heat shock protein synthesis rises transiently, reaching a maximum after 5–7 min, and then decreases to a new steady-state rate after 15–20 min [for review, see Neidhardt et al. 1987]. Perhaps a temperature increase results in the unfolding of a set of proteins that act as a transient signal for HSP induction and then are proteolyzed rapidly by the degradative systems of the cell. In contrast, the increased synthesis of heat shock proteins that follows expression of the plasmid-borne unfolded protein studied here is not transient [see Figs. 2 and 5]. Synthesis of heat shock proteins remains high at
least 60 min after addition of IPTG (the latest time measured). In this case, increased HSP synthesis presumably persists because of the continuous overproduction of unfolded protein, the inducing signal.

Studies by Bahl et al. (1987) report immediate growth inhibition after induction of heat shock proteins by expression of the \( \lambda \) cIII protein. They suggest that a prolonged heat shock response is deleterious and that the transient nature of the normal response may be essential (Bahl et al. 1987). In contrast, we found no significant effect on growth rate during the 2 hr after heat shock protein synthesis was induced by unfolded protein. Therefore, it appears that prolonged expression of heat shock proteins is not immediately deleterious. Perhaps the growth inhibition observed with cIII is a result of some other aspect of its overexpression.

How might unfolded protein function in vivo as a signal for induction of the heat shock response? An increase in the concentration of \( \sigma^{22} \), resulting from both a decrease in its degradation rate and an increase in its synthesis rate, is thought to be directly responsible for the increased level of heat shock protein synthesis seen during a heat shock (Straus et al. 1987). It has been suggested (Goff and Goldberg 1985; Straus et al. 1987; Goff et al. 1988) that unfolded proteins generated during a heat shock stabilize \( \sigma^{22} \) indirectly by competing for intracellular proteases, such as protease La, and thus decrease the degradation of \( \sigma^{22} \). Our results suggest that if, indeed, this mechanism is operative, then unfolded proteins do not need to be degraded rapidly to compete for proteases. Moreover, our results show that protease La is clearly not required for the observed heat shock response.

We would like to suggest another possible mechanism for the induction of HSPs by the presence of unfolded protein. Namely, unfolded protein may bind to \( \sigma^{22} \) directly and thereby affect its stability or activity, or it may affect an earlier step in the signal transduction process by binding to another protein that influences \( \sigma^{22} \) activity. There is precedent for the idea that the action of a \( \sigma \) factor can require other proteins and that environmental stress signals can act to regulate the activity of these proteins. For example, in enteric bacteria nitrogen starvation results in the phosphorylation of a regulatory protein that acts in concert with \( \sigma^{54} \) to activate transcription from nitrogen-regulated promoters (for review, see Magasanik 1988).

Methods

Nomenclature

In this work two variants of the amino-terminal domain of \( \lambda \) repressor are used. The variant designated N\(^{92} \) contains the first 92 residues of the \( \lambda \) repressor [Sauer and Anderegg 1978]. The N\(^{102LT} \) variant contains the amino-terminal 102 residues of \( \lambda \) repressor fused to the LT ('long tail'), sequence \([NH_2-Lys-Val-Glu-Ala-Pro-Thr-Ala-Val-Thr-Arg-Ala-Val-Val-Lys-Leu-Glu-Lys-Asn-Gln-His-Glu-COOH] \), described by Bowie and Sauer [1989]. When the LT sequence is added to the carboxyl terminus of bacteriophage P22 Arc repressor, it stabilizes Arc against proteolysis in vivo, apparently by affecting the suitability of the protein as a substrate for proteases [Bowie and Sauer 1989]. This extension also has been shown to protect mutants of the amino-terminal domain of \( \lambda \) repressor from intracellular degradation [Bowie and Sauer 1989, this paper].

Bacterial strains and plasmids

The following \( E. coli \) K-12 strains were used in this work. Strain DP700 is ara \( \Delta \{lac \} \) naiA argEam rif thi-1/F' lacQ lacZ::Tn5[Kan\(^R \)] p+ and is lysogenic for a \( lambda \) strain, which carries a \( P_{bopZ-} \)lacZ fusion (a gift from Dr. J. Wild). This strain phenotypically is \( lacZ^{-} \). Strain DP700 ion\(^- \) was constructed by P1 transduction [Miller, 1972] of the ion::\( \Delta \)Tn10 allele [Maurizi et al. 1985]; strain CAG2039 [a gift from C. Gross] is ara \( \Delta \{lac \} \) thr tpi pam supCts htpR165am/F' lacQ lacZ::Tn5, strain CAG2041 [Grossman et al. 1987] is CAG2039 htpR\(^{+} \).

The plasmids that encode the \( \lambda \) repressor amino-terminal domain proteins are derivatives of plasmid pRB104. This is a pBR322 derivative containing an M13 origin of replication and a gene encoding residues 1–102 of \( \lambda \) repressor under transcriptional control of a tac promoter [Breyer 1988]. The construction of the plasmid encoding the protein LA57-N\(^{92} \) has been described previously [Parsell and Sauer 1989]. The plasmid encoding the protein N\(^{102LT} \) was a gift from J. Bowie. The plasmid encoding the protein LA57-N\(^{102LT} \) was constructed by ligating the 3.5-kb \( Psil-EcoRI \) fragment of the N\(^{102LT} \) plasmid with the 1-kb EcoRI–\( Psil \) fragment of a plasmid encoding the LA57-N\(^{102} \) protein [Parsell and Sauer 1989], using standard techniques [Maniatis et al. 1982]. To confirm that the constructions were correct, single-stranded plasmid DNA was purified.
from an M13RV1 transducing lysate [Vershon et al. 1986], and DNA sequences were determined using the dyeoxy method [Sanger et al. 1977].

**Buffers**

Standard buffer (SB100) is 10 mM Tris-HCl (pH 8), 2 mM CaCl₂, 0.1 mM EDTA, 100 mM KCl, and 5% glycerol (vol/vol) [SB15 and SB50 contain 15 and 50 mM KCl, respectively]. Lysis buffer is 100 mM Tris-HCl (pH 8), 1 mM EDTA, 10 mM 2-mercaptoethanol, 500 mM NaCl, 6 mM urea, and 0.1% NP-40 (vol/vol).

**Protein purification**

Wild-type and mutant variants of the amino-terminal domain were purified from E. coli strain CAG2039 [relevant genotype htpR⁺], bearing appropriate overproducing plasmids, using a modification of the general method of Sauer et al. [1986]. Cells were sonicated in cold lysis buffer, polyethyleneimine was added to 0.5% [from 10% stock [vol/vol] at pH 8], and the sonicate was stirred at 4°C for 10 min. After centrifugation, the supernatant was taken and proteins were precipitated by addition of ammonium sulfate to saturation. The ammonium sulfate pellet was resuspended in SB100 plus 6 mM urea and was dialyzed using Spectrapor 3 tubing against SB100. Precipitated protein was removed by centrifugation, and the supernatant was diluted to 50 mM KCl prior to loading onto a Bio-Rad Affigel Blue column [for LA57-N102LT] or a CM-Sephadex column [for N102LT]. Peak fractions were collected and run on a Sephadex G75 [superfine] column. Peak fractions from this column were concentrated using an Amicon YM5 filter. The LA57-N102LT protein fractions from the G75 column were purified with one step further by dialysis into SB15 and chromatography on a CM-Sephadex column.

**Circular dichroism**

Circular dichroism measurements from 250 to 203 nm were taken using an AVIV model 60DS spectropolarimeter. Melting curves were obtained by measuring [θ]₂₂₂ as a function of temperature as described by Hecht et al. [1984]. For these studies protein concentrations were 50 μg/ml in 10 mM potassium phosphate (pH 7.0), 100 mM KCl.

**Pulse-chase experiments**

Cultures of E. coli strain DP700, containing plasmids encoding wild-type or mutant variants of the amino-terminal domain, were grown at 37°C to an OD₆₀₀ of 0.2-0.3 in minimal medium as described for pulse-chase experiments. Transcription from the P₆₅₉ promoter was induced by the addition of 1 mM IPTG. At intervals after addition of IPTG, 1-ml aliquots were removed to Falcon 2059 tubes at 37°C and pulse-labeled by addition of 37.5 μCi of L-[³⁵S]methionine [sp. act. >800 Ci/mmmole] per milliliter of cells. After 2 minutes, incorporation of labeled methionine was stopped by transferring 0.5-ml portions of cells to Eppendorf tubes that contained 25 μl of unlabeled methionine [28 μg/μl] and 35 μl of the protease mixture used in pulse-chase experiments. When synthesis of HSPs was monitored as a function of IPTG concentration, cultures were induced with IPTG for 30 min prior to pulse-labeling. Samples were processed as described for pulse-chase experiments, and lysates were loaded on 7.5% polyacrylamide gels [Laemmli 1970]. Gels were autoradiographed, and band intensities were measured as described above.

Induction of heat shock proteins after a temperature increase was assayed as described above, except that IPTG was not added and cells were transferred to 42°C for 7 min prior to labeling with L-[³⁵S]methionine.

**Western blot**

Estimates of the amount of LA57-N102LT protein present in DP700 cells after a 30-min induction with various amounts of IPTG were obtained using Western blot analysis. Lysates from HSP induction experiments were electrophoresed on 15% polyacrylamide gels [Laemmli 1970], and Western blots were performed using polyclonal antiserum raised against the amino-terminal domain of λ repressor [Breyer 1988], essentially as described [Ausubel et al. 1989]. Horseradish peroxidase conjugated donkey anti-rabbit IgG was used as a secondary antibody.

**Acknowledgments**

We thank Jadwiga Wild for providing the P₆₅₉-lacZ fusion, Carol Gross for supplying strains CAG2039 and CAG2041, Rich Breyer for plasmid pBR104 and antiserum against the amino-terminal domain, Jim Bowie for the plasmid encoding the N102LT protein, Peter Kim for use of the spectropolarimeter,
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and Jim Hu and Alan Grossman for critical reading of the manuscript. Discussions with Alan Grossman, Jim Hu, Jim Bowie, and David Straus contributed greatly to the progress of this work. This work was supported by a National Institutes of Health grant 16892 and by a predoctoral grant (to D.A.P.) from the Keck Foundation.

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*Genes Dev.* 1989, 3:
Access the most recent version at doi:10.1101/gad.3.8.1226