A large decrease in heat-shock-induced proteolysis after tryptophan starvation leads to increased expression of phage λ lysozyme cloned in *Escherichia coli*

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The R gene coding for phage λ lysozyme (λ L), cloned under the control of the P_L promoter on a multicopy vector, is expressed in an *Escherichia coli* strain auxotrophic for tryptophan. Induction by a thermal shift after tryptophan supplementation in a culture initially brought into stationary phase by tryptophan starvation leads to highly increased expression. A thermally unstable mutant protein, difficult to obtain under standard conditions, can be easily produced by post-stationary-phase expression. It is shown that this is due to a drastic decrease in the heat-shock-induced proteolysis normally observed on thermal induction. These data are discussed in relation to our present knowledge of stringent and heat-shock responses.

INTRODUCTION

Protein biochemistry has been revolutionized by the ability to overexpress cloned genes. High levels of expression of foreign genes in *Escherichia coli* is now frequently achieved. The problems that remain are: the design of optimal ribosome-binding sites, the avoidance of proteolysis and the production of the proteins in a native state [1].

The initial motivation for the work described here was an interest in the incorporation of amino acid analogues into proteins for enzymological and biophysical studies. This work was planned for phage λ lysozyme (λ L), a small protein of medium thermal stability, the gene for which had been cloned and efficiently expressed in a plasmid vector based on the widely used λP_L promoter [2]. Initially, we wanted to incorporate a tryptophan analogue at a high level. This led us to modify the standard expression conditions described previously. The expression was carried out with an *E. coli* strain auxotrophic for tryptophan; the culture was initially starved of tryptophan and supplemented with this amino acid or its analogue at the time of induction. Unexpectedly, under these conditions, a greatly increased level of expression was observed. The origin of the hyperexpression was then investigated, particularly the effect of the expression conditions on intracellular proteolysis.

The protocol that we use combines conditions leading to the stringent response (for reviews, see refs. [3] and [4]) and the heat-shock response (for reviews, see refs. [5–9]). A tentative interpretation of our observations in terms of what is known about these two physiological responses is proposed.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *E. coli* strain M5219 (λ cI_{857} lysogene, trp_{am}) [10] was transformed with the plasmids pLJ0516, pAP68 or pPS01. These contain the R gene coding for λ L downstream from the P_L promoter; the expression is under the control of the thermolabile

cI_{857} repressor. In plasmid pLJ0516, the ribosome-binding site has been optimized; in plasmid pAP68, a bicistronic construction is used and the ribosome-binding site is the natural one [2]. Plasmid pPS01 is similar to pLJ0516 but it encodes a mutant lysozyme H31D obtained by site-directed mutagenesis (P. Soumilion & J. Fastrez, unpublished work). It was checked that M5219 is $RelA^+$ by testing its serine-sensitivity [11]: this strain grows on minimal plates supplemented with tryptophan and serine (100 μ g/ml each).

Expression of λ L

Strains harbouring plasmids were grown at 30 °C in two different media. In the LB medium (1 % tryptone, 0.5 % yeast extract, 0.1 M-NaCl) supplemented with ampicillin (100 μ g/ml), cultures were shifted to 42 °C when the A_{570} reached 0.6; expression of λ L was induced for 2 h. CAS medium was M9 medium [$Na_2HPO_4 \cdot 7H_2O$ (6.4 g/l, 0.048 M), KH_2PO_4 (1.5 g/l, 0.022 M), NaCl (0.25 g/l, 0.009 M) and NH_4Cl (5 g/l, 0.019 M)] [12] supplemented with casamino acids, an acid-hydrolysed casein devoid of tryptophan (Difco Laboratories, Detroit, MI, U.S.A.) (12 g/l), ampicillin (100 μ g/ml), L-tryptophan (2 μ g/ml), $CaCl_2$ (0.1 mM) and $MgCl_2$ (1 mM). Cultures starved of tryptophan reached a stationary phase at approx. 1.2 A_{570} . At different times before and during this stationary phase, the cultures were shifted to a temperature of 42 °C and L-tryptophan was simultaneously added (50 μ g/ml). Expression of λ L was induced for 75 min.

The percentage of λ L in relation to the total protein was estimated in two ways. First, the proteins were analysed on 0.1 % (w/v) SDS/15 % (w/v) polyacrylamide gels [13]. After being stained with Coomassie Brilliant Blue R250 and dried, the gels were scanned in a Gilford Response II spectrometer. In the second method, total protein content was first determined: 1 ml of culture at an A_{570} around 2 was centrifuged, resuspended in 200 μ l of 1 M-Tris (pH 7.0)/0.1 M-EDTA for 15 min. After a second centrifugation and suspension in 500 μ l of water, the cells lysed spontaneously; 50 μ l of 50 % (v/v) trichloroacetic acid was added. The precipitated proteins were pelleted and resuspended in 200 μ l of 5 % (w/v) SDS. After being heated for 10 min at 100 °C, samples were analysed by the bicinchoninic acid method

Abbreviations used: λ L, phage λ lysozyme; hsp, heat-shock protein; T_m , temperature of half-thermal denaturation.

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[14]. A solution of BSA (1 mg/ml) in 0.15 M-NaCl (supplied by Sigma) was used as reference. Further, the λ L concentration was determined by measuring the activity present in the sample after lysis by CHCl_3 treatment (3%) [15] and comparing it with a calibration curve established with pure λ L. Strain MM294 [16] was used to measure the activity as described [2].

Measurement of intramolecular λ L stability

By a pulse-chase experiment. Two cultures of M5219-pLJ0516 were grown in CAS medium at 30 °C and expression was induced respectively during the exponential and stationary phase (0.6 and 1.2 A_{570}). Induction was carried out by shifting the cultures to a temperature of 42 °C and adding radioactive L-tryptophan (^{14}C -labelled on the β -carbon; Amersham) at a final concentration of 2.5 $\mu\text{g/ml}$ and 20 $\mu\text{Ci/mg}$. After 15 min, concentrated non-radioactive tryptophan (2 mg/ml) was added to a final concentration of 50 $\mu\text{g/ml}$. Radiolabelled λ L was followed at different times after chase by autoradiography (Amersham β -Max hyperfilm) and liquid-scintillation counting (the gels, wet or dried, were sliced and counted in 5 ml of Aqualuma).

By blocking protein synthesis. Cultures of M5219-pLJ0516 were rapidly brought to a temperature of 42 °C for 15 min so that an appreciable amount of λ L was synthesized. Then chloramphenicol (500 $\mu\text{g/ml}$) and sodium azide (0.5%) were added to block protein synthesis and bacterial growth. λ L activity was then measured as a function of time.

RESULTS

Experiments were first carried out to follow the growth of the M5219 strain auxotrophic for tryptophan and harbouring plasmid pLJ0516 in CAS medium at 30 °C under conditions of tryptophan limitation (2 $\mu\text{g/ml}$). Starting from a culture with an initial A_{570} around 0.12, the growth curve was that shown in Fig. 1(a) (solid line). When λ L was expressed after a stationary-phase period by shifting the temperature to 42 °C immediately after tryptophan supplementation, a greatly increased expression was observed.

In order to determine the point where hyperexpression appears, inductions were started at different times before or during the stationary phase arising from tryptophan starvation. After expression of the R gene for 75 min, the total protein content of 1.5×10^7 cells from each culture was analysed by SDS/PAGE. The results are shown in Fig. 1(b); also shown is the result obtained when the expression was induced during exponential growth in LB medium (lane A). In CAS medium, the increased expression appears progressively with the entry of cells into the stationary phase; it is particularly marked after 300 min (Fig. 1b, lanes D, E and F) and remains marked for at least 3 h thereafter, although at decreased levels. After 300 min, the viability of the cells become progressively more compromised; after 500 min, growth is strongly inhibited and some of the cells become elongated; this suggests that septation is inhibited.

The expression levels achieved after a stationary phase are compared in Table 1 with those obtained when the induction was carried out during exponential growth in LB medium or CAS medium supplemented with tryptophan. As previously reported [2], NaCl addition up to 0.4 M to an LB medium improves the apparent expression induced during the exponential phase by about a factor of 3 in terms of the percentage of total protein. In CAS medium, the expression is also higher than in the LB standard medium. Finally, in LB medium supplemented with the salts of M9 medium (composition given in the Materials and

Fig. 1. Expression of λ L during exponential growth and after tryptophan-starvation periods of various length

(a) Progress curve of the A_{570} of 50 ml cultures of M5219 harbouring the pLJ0516 plasmid at 30 °C in LB (---) and CAS medium (—), and after induction of λ L expression by a temperature shift to 42 °C (···); for the CAS medium, tryptophan was added at a final concentration of 50 $\mu\text{g/ml}$ on temperature shift. (b) SDS/15% polyacrylamide gels stained with Coomassie Brilliant Blue R250. Lanes A–G: protein content of the cells collected after λ L induction at the points indicated in (a); for each lane, 1.5×10^7 cells were loaded; lane H, purified λ L (1 μg).

methods section), the final λ L yield is similarly improved. This is presumably due to the effect of the salts present in CAS medium. In a post-stationary-phase induction, the λ L yield is further increased by a factor of 2–3. When the induction is initiated at 300 min, the maximum yield is reached after 75 min at 42 °C and does not rise thereafter. Post-stationary-phase expression from plasmid pLJ0516 is unaffected by NaCl (results not shown). Under all conditions, the λ L remains soluble in the cells.

An increased yield is also observed when λ L is expressed from another plasmid, pAP68. The level of expression observed with this plasmid is always lower than that obtained with pLJ0516

Table 1. Expression of the R gene coding for λ L under different conditions

The expression of the R gene coding for λ L is initiated under various conditions: (a) LB-Exp: in standard LB medium during exponential growth at 30 °C to an A_{570} of 0.6 by induction at 42 °C for 3 h; (b) LB-Exp-NaCl: in LB medium but with 0.4 M-NaCl following the same protocol as in (a); (c) CAS-Exp: the culture is grown on CAS medium containing 3 μ g of L-tryptophan/ml at 30 °C up to an A_{570} of 0.6, then induced at 42 °C for 3 h on supplementation with 50 μ g of L-tryptophan/ml; (d) CAS-Stat: in CAS medium as in (c) except that 2 μ g of L-tryptophan/ml is initially added; after a stationary phase at A_{570} 1.2 (300 min), the induction is run for 75 min on supplementation with 50 μ g of L-tryptophan/ml. The λ L quantity obtained per litre of culture is determined from activity measurements and from SDS/PAGE, different quantities of pure λ L being added in several lanes as references. The percentage of λ L versus total protein is determined from the numbers given in column 3 and the total protein content measured by the bicinchoninic acid method. Values estimated from scanning the gels, as in ref. [2], are about twice as high.

Conditions	Final A_{570}	λ L (mg/l)	Percentage of total protein
LB-Exp	2.6	10	2.7
LB-Exp-NaCl	1.8	20	8
CAS-Exp	2.7	30	8
CAS-Stat	2.4	70	24

because the ribosome-binding site that controls translation is less efficient [2]. Here, a fivefold increase in yield is also found for post-stationary-phase expression in CAS medium over exponential-phase expression in LB medium. NaCl addition in post-stationary-phase expression with this plasmid decreases the λ L yield (results not shown).

In previous work on the optimization of λ L expression [2], we have shown that NaCl addition improves the apparent expression because it slows down proteolysis. The absence of a positive NaCl effect in the new conditions led us to suspect that a decrease in the rate of degradation *in vivo* could again explain the increased expression. λ L is a protein of medium thermal stability. *In vitro*, its temperature of half-denaturation (T_m) at pH 7.5 is around 52 °C and it may be partially unfolded at 42 °C. Unfolded proteins are known to be degraded faster by proteolysis than native ones. Indeed, we have shown previously that λ L is susceptible to proteolytic cleavage *in vivo* after a temperature shift to 42 °C. Besides, this shift is known to lead to the heat-shock response which induces the synthesis of proteinases.

The hypothesis that a large decrease in proteolysis of λ L was responsible for the yield increase was checked by a pulse-chase experiment. λ L was expressed for 15 min in the presence of 14 C-labelled tryptophan and, after addition of excess non-radioactive tryptophan for another 170 min, labelled λ L was followed by SDS/PAGE as a function of time. The results are shown in Fig. 2. Both in post-exponential growth at 30 °C and in post-stationary phase, the total λ L concentration increases with time, but significantly more in the second protocol (Fig. 2a). The labelled λ L concentration, on the other hand, clearly decreases faster with time in the first protocol than in the second (Fig. 2b). The rates of proteolysis have been measured by scanning the autoradiographs of the gels and by liquid-scintillation counting. Half-lives of 77 ± 13 min and greater than 150 min respectively have been obtained. The stability increases with the length of the stationary phase before induction; the other labelled proteins are less affected (Figure 2b).

To rule out the hypothesis that the apparent increase in expression could be due to better translation, the intracellular

stability of λ L was also measured by following its activity after inhibition of protein synthesis. The data are shown in Fig. 3. The half-life in CAS medium is increased from 73 to 217 min if the culture has been through a stationary phase.

As part of an investigation of the role of the histidines in the stability and activity of λ L, His-31 was mutated to aspartic acid. This protein is fully active but it is more easily denatured than the wild-type enzyme, its T_m *in vitro* being lower by 7 °C (P. Soumilion & J. Fastrez, unpublished work). The percentage of the protein that is unfolded at 42 °C should accordingly be higher for the H31D mutant than for the wild-type enzyme. Proteins being more susceptible to proteolysis in their unfolded form, it is expected that, under conditions where proteolysis is effective, the apparent yield of the H31D mutant should be significantly lower than that observed for the wild-type enzyme. Indeed, the expression of the temperature-sensitive allele of λ L from the M5219-pPS01 clone leads to disappointing results when initiated during exponential growth (Fig. 4, lane 2). Its post-stationary-phase expression gives a dramatically improved yield (Fig. 4, lane 4). When expressed at this high level, the protein remains soluble.

Fig. 2. Expression and proteolysis of λ L observed after exponential growth or a period of tryptophan starvation at 30 °C

(a) SDS/15% polyacrylamide gel stained with Coomassie Brilliant Blue R250 showing total protein. Lanes 1–5, after exponential growth in CAS medium to an A_{570} of 0.6 at 30 °C, the λ L expression was induced by a temperature shift to 42 °C while radioactive tryptophan was added (2.5 μ g/ml); non-radioactive tryptophan was added 15 min later (50 μ g/ml). The cells were collected 0, 20, 50, 110 and 170 min after the addition of the non-radioactive tryptophan. Lanes 6–10, after a stationary phase in CAS medium (300 min at 30 °C), λ L expression was induced and the cells were collected as above. (b) Autoradiographs of the same gels as above but not stained. For each lane, 80 μ l of culture were loaded.

Fig. 3. Decrease in λ L activity observed after inhibition of protein synthesis

The expression of the gene coding for λ L was initiated in several media; after 15 min, chloramphenicol (500 μ g/ml, final concn.) and sodium azide (0.5%) were added and λ L activity was measured as a function of the time elapsed after inhibition of protein synthesis; the points are experimental, the lines are fitted exponentials. CAS medium during exponential growth ($\blacksquare$, half-life = 73 min), CAS medium after a stationary phase ($\square$, half-life = 217 min) or standard LB medium during exponential growth (----) (line calculated from the data reported in ref. [2], half-life = 37 min).

Fig. 4. Comparative expression of wild-type λ L and the H31D temperature-sensitive mutant after exponential growth or stationary phase

SDS/15% polyacrylamide gel of the protein content of M5219 cells harbouring the pLJ0516 and pPS01 plasmids coding respectively for the wild-type and mutant lysozymes. Lane 1, expression of wild-type λ L in LB medium supplemented with NaCl (0.4 M) during exponential growth (induction by temperature shift to 42 °C at $A_{570} = 0.6$ for 2 h). Lane 2, expression of mutant λ L under identical conditions. Lane 3, expression of wild-type λ L induced by temperature shift to 42 °C and tryptophan addition (50 μ g/ml) for 75 min after a stationary phase in CAS medium (300 min at 30 °C). Lane 4, expression of mutant λ L under identical conditions. For each lane, 50 μ l of culture was loaded. Lane 5, purified λ L (1 μ g).

DISCUSSION

The results presented here clearly show that, in the post-stationary phase expression at 42 °C, the proteolysis observed on induction during exponential growth of λ L is considerably suppressed. Consequently, after only 75 min of induction high levels of expression are observed and an unstable protein can be prepared in high yield. Our observation might be of biotechnological interest and is relevant to current active investigations on stress responses.

We have shown previously that λ L is rapidly degraded *in vivo*, its unfolded state being an intermediate at 42 °C [2]. The intracellular stability was first measured by following the intensity of the band corresponding to λ L on SDS/polyacrylamide gels as a function of time after inhibition of protein synthesis: it decreased relatively rapidly showing that the protein was degraded. Then λ L activity remaining in cells in which protein synthesis had been blocked and which were incubated at several temperatures was followed as a function of time. The half-life of the disappearance of activity was shown to be extremely sensitive to the temperature of incubation. This was interpreted on the basis of the following scheme:

where N and D stand for the native and denatured forms of the protein, K_D and k_{prot} stand for the reversible denaturation equilibrium constant and proteolysis rate constant respectively. This scheme accounts for the large temperature effect on the rate of proteolysis because the equilibrium constant of reversible denaturation is very temperature sensitive as a consequence of the large enthalpy of denaturation. It was shown also that the addition to the medium of dichloroisocoumarol, a specific inhibitor of the La proteinase, increases the half-life of λ L; this result suggested that this proteinase contributes very significantly to the degradation.

A sudden temperature shift is known to induce a transient response leading to the increased production of at least 17 heat-shock proteins (hsps) [6]. A significant rise in proteolysis is observed, as a result of the increased expression of the *lon* gene product also referred to as proteinase La [17–20]. Besides Lon, however, other proteinases or hsps are probably working in concert as a degradation machinery [21,22]. The production of unstable, unfolded or abnormal proteins also induces Lon expression [23].

Solutions have been proposed to the inconvenience represented by proteolysis. In a genetic approach [24], Lon mutants have been used, but their unwanted phenotypes, such as capsid overproduction or u.v. sensitivity, have to be suppressed by further mutations. A 50% decrease in proteolysis results. Mutants deficient in heat-shock response as a consequence of a nonsense mutation in their σ^{32} factor (htpR165 [19,25]) can also be used; they are viable at 30 °C because of the presence of a temperature-sensitive suppressor; at 42 °C, the proteolysis is more strongly suppressed than in Lon mutants but the cells die rather quickly. Variations in culture conditions such as NaCl addition can also significantly decrease proteolysis [2]. In the work described here, cell culture conditions suppress proteolysis nearly as efficiently as with the htpR165 strain; the cells remain viable if expression is induced at the optimal time of the stationary phase.

The interpretation of these observations in terms of our present understanding of the stringent and heat-shock responses is not easy. The hsps described above are under the control of several promoters, one of which is recognized by a form of the RNA polymerase in which the normal σ^{70} factor is replaced by a σ^{32} (product of the *htpR* gene), the heat-shock regulatory factor [26,27]. σ^{32} is a very unstable protein ($t_{1/2} \cong 0.7$ –1.0 min at 42 °C) [28,29]. On subjection to heat shock, its concentration increases

transiently, and, after an overshoot, reaches a new steady state through feedback control [28–31]. There is an increased transcription of the *hspR* gene itself, under the control of one of its promoters [32] recognized by a thermostable σ^{24} factor [33] (also called σ^E [34]). The kinetics of the overshoot in σ^{32} after 10 min, however, is explained mainly by the transient increase in its translation and by its transient stabilization (slow degradation for 5 min) [28,35]. The mechanism of the translational increase is not fully understood. The stabilization of σ^{32} has been attributed to the fact that, on subjection to heat shock, many unstable proteins are denatured and compete for the proteinase La involved in degradation [21]. More recently, an attractive hypothesis has been proposed in which the necessity of a physiological response is sensed by the degree of saturation of a thermometer protein (DnaK in *E. coli*) complexing unfolded proteins. The σ^{32} factor is also complexed by DnaK and this increases its susceptibility to proteolysis. On heat shock, DnaK is saturated by unfolded proteins and σ^{32} is then stabilized. This phenomenon is transient because DnaK is a hsp positively regulated by σ^{32} [9].

Under conditions of amino acid starvation, complex physiological changes appear, i.e. the stringent response. The response is observed in all strains except those lacking functional enzymes involved in the handling of 3'-diphosphoguanosine 5'-diphosphate; these strains show an abnormal behaviour (RelA⁻ strains have been particularly investigated) [3,4]. Under the conditions of the stringent response, proteolysis is increased [36–38], and many unstable proteins are degraded to release the amino acids necessary for survival. Some of the hsps, including proteinase La, have been shown to be overproduced; however, this aspect of the stringent response appears to depend on the inducing conditions: it is observed (only in RelA⁺ strains) as a consequence of the denaturation of a thermolabile valyl-tRNA synthetase [39] and not on amino acid starvation or repression of leucine synthesis [21,33,40,41]. Under the conditions of the stringent response, the σ^{24} responsible for the increased transcription of the *hspR* gene during heat shock disappears [33]. Tryptophan starvation induces this stringent response [42–47], although the effect on proteolysis [45] and polyribosome turnover [46] might be lower.

The disappearance of σ^{24} during amino acid starvation could explain the apparent absence of heat-shock-induced proteolysis. As mentioned above, however, a change at the transcriptional level is not the main origin of the response. Alternatively there could be an indirect effect of the starvation on the transient σ^{32} stabilization: as a consequence of the stringent response, the unstable proteins disappear; on temperature shift, the proteinase La or the DnaK thermometer would then fail to be saturated by unfolded proteins, and consequently, σ^{32} would not be protected from proteolysis and the heat-shock response would not be induced. It is, however, not clear why heat-shock proteolysis does not seem to occur after some time. This may be related to the transient nature of the heat-shock response; it may be that it is induced only when unfolded proteins are suddenly produced in large amounts; this would not happen here. A firm answer to these questions will require more studies.

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