Effect of Temperature on In Vivo Protein Synthetic Capacity in *Escherichia coli*

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In this report, we examine the effect of temperature on protein synthesis. The rate of protein accumulation is determined by three factors: the number of working ribosomes, the rate at which ribosomes are working, and the rate of protein degradation. Measurements of RNA/protein ratios and the levels of individual ribosomal proteins and rRNA show that the cellular amount of ribosomal machinery in *Escherichia coli* is constant between 25 and 37°C. Within this range, in a given medium, temperature affects ribosomal function the same as it affects overall growth. Two independent methodologies show that the peptide chain elongation rate increases as a function of temperature identically to growth rate up to 37°C. Unlike the growth rate, however, the elongation rate continues to increase up to 44°C at the same rate as between 25 and 37°C. Our results show that the peptide elongation rate is not rate limiting for growth at high temperature. Taking into consideration the number of ribosomes per unit of cell mass, there is an apparent excess of protein synthetic capacity in these cells, indicating a dramatic increase in protein degradation at high temperature. Temperature shift experiments show that peptide chain elongation rate increases immediately, which supports a mechanism of heat shock response induction in which an increase in unfolded, newly translated protein induces this response. In addition, we find that at low temperature (15°C), cells contain a pool of nontranslating ribosomes which do not contribute to cell growth, supporting the idea that there is a defect in initiation at low temperature.

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*Escherichia coli* cells will grow over a temperature range of about 40°C, and remarkably, the cell growth rate increases in response to increasing temperature like a simple chemical reaction in a central normal range of its growth temperatures (20 to 37°C). At both high and low temperatures, this relationship breaks down. It is thought that at low temperature, protein initiation becomes rate limiting for growth (3, 6), but it is not known what is rate limiting at high temperature. We have examined one important aspect of cell physiology, translation, as a step toward understanding the effect of temperature on cell growth.

It is known from studies of cell growth in different media that *E. coli* tightly controls the levels of the protein synthesizing machinery. At different growth rates at the same temperature, the rate at which ribosomes are working (peptide chain elongation rate) is fairly constant, but the number of ribosomes per cell increases markedly with increasing growth rate (15, 21). Thus, at high growth rates many ribosomes are working whereas at lower growth rates fewer ribosomes are working, but the ribosomes in both cells (growing at different rates) are working at approximately the same rate. It should be noted that there is also an increase in peptide chain elongation rate at high growth rates which may have important regulatory functions, but the change in protein synthetic capacity is primarily due to alterations in the levels of ribosomes (21). Thus, we know that cells change the number of ribosomes per cell to increase their growth rate in response to richer medium, but how do cells regulate the protein synthetic machinery in response to temperature changes? We can answer this question by determining the number of ribosomes per cell (and the fraction actually engaged in protein synthesis) and the peptide chain elongation rate; together these factors determine the protein synthetic capacity.

Previous work in this lab and others has shown that the levels of ribosomal protein and RNA are constant between 23 and 42°C in a given medium. Herendeen et al. (12) have used two-dimensional gel electrophoresis to measure the steady-state levels of ribosomal proteins in cells grown at various temperatures in glucose-rich medium. Their results show that there is only small variation in the levels of individual ribosomal proteins in cells growing in this range and that as a group they neither increase nor decrease with increasing temperature. In addition, Ryals et al. (28) have shown that the level of rRNA is constant over this temperature range.

We have determined the polypeptide chain elongation rate throughout the range of *E. coli* growth temperatures as well as after a shift to high temperature. In addition, we have confirmed that the level of ribosomal components is constant between 37 and 42°C and have examined the polysome profiles of cells growing at 15, 37, and 42°C. The results show that peptide chain elongation rate increases as a function of temperature. In the normal temperature range, the rate of peptide chain elongation matches the growth rate, but at both high and low temperatures there appears to be an excess of protein synthetic machinery. At low temperature this may indicate a defect in initiation of translation, but at high temperature there is not an excessive pool of nontranslating ribosomes. This indicates that protein degradation occurs at a much higher rate at the higher temperature.

**MATERIALS AND METHODS**

Strains and growth conditions. *E. coli* W3110 (prototrophic) was used in all experiments. Cells were grown in MOPS (N-[1-morpholine]propanesulfonic acid) medium (20) supplemented with 0.4% glucose, 20 amino acids, vitamins, and bases as previously described (36). Medium for amino acid labeling experiments was the same except leucine and methionine concentrations were lower (80 and 20 μM, respectively, instead of 500 and 200 μM) and uridine was omitted when labeling was with radioactive uridine. Cells were grown aerobically in a...
Peptide chain elongation rate as determined by β-galactosidase induction time. The most obvious reason for cell growth rate to increase in response to temperature is that the rate of polysome elongation may increase as a function of temperature, identical to the growth rate. We first wanted to determine if this was true within the normal temperature range (25°C to 37°C) and then see what happens outside the normal range (i.e., at 42°C and at 10 to 20°C).

Measurements of β-galactosidase induction time were done at a number of temperatures both within the normal temperature range and at higher temperatures. When the elongation rate is plotted along with the specific growth rate on an Arrhenius plot (Fig. 2A), two results become clear. First, peptide chain elongation rate increases in proportion to growth rate in the normal range of temperatures. Thus, cells growing in this temperature range have no need for an increased number of ribosomes; their ribosomes are simply able to translate faster as the temperature increases. The second result was more surprising: peptide chain elongation rate increases in proportion to increasing temperature even at high temperature (37°C to 44°C) when growth rate changes very little. This result is also illustrated in a plot of growth rate versus elongation rate (Fig. 2B). In the normal temperature range, growth rate is proportionally to elongation rate, but at high temperature growth rate…

Peptide chain elongation rate measured by pulse-labeling experiments. In a second method to measure the elongation rate (2, 7), cells are pulsed with a radioactive amino acid ([3H]leucine) for a short time (5 to 15 s) and then chased with an unlabeled amino acid. The pulse is so short that only a fraction of each polypeptide being translated at the time is labeled. During the chase, samples are taken periodically to measure radioactivity in completed protein. Radioactivity is chased into completed protein until the polypeptide initiated during the pulse (and thus with only amino acids near its N terminus labeled) is finally completed and no more radioactivity is chased into the completed protein. The time when this occurs is the translation time of the protein. Radioactivity in completed protein is followed by separating proteins by two-dimensional gel electrophoresis and determining the amount of radioactivity in individual protein spots. A control culture labeled with another isotope ([35S]methionine) is mixed with the sample to control for sampling error.

Cells were pulse-labeled for 15 s with 0.25 mCi of [3H]leucine (140 Ci/mmol; 5 mCi/ml; ICN) per ml and chased with 6.7 mM cold leucine. Then 0.5-ml samples were placed in Eppendorf tubes containing 0.5 ml of partially frozen MOPS buffer containing 400 μg of chloramphenicol per ml and 20 mM sodium azide; 0.2 ml of [35S]methionine-labeled cells was added to each sample. Cells were labeled for three to four generations in the presence of 50 μg/ml of [35S]methionine (1,100 Ci/mmol, 11 mCi/ml; ICN) per ml. Labeling was terminated by adding an equal volume of MOPS buffer containing 400 μg of chloramphenicol per ml and 20 mM sodium azide, and samples were placed on ice until mixed with the [3H]leucine-labeled samples.

Two-dimensional gel electrophoresis and measurement of radioactivity in protein spots. Two-dimensional gel electrophoresis was performed as previously described (1, 23, 35). Extracts were made, and half was placed on each of two gels. These duplicate gels were stained with Coomassie blue and dried, and autoradiograms were prepared. Spots were excised and prepared for quantification. In addition, as described previously (24), trichloroacetic acid precipitation was done to obtain the [3H]/[35S] ratio in each sample extract.

Calculation of radioactivity for pulse-chase experiments. The radioactivity in completed protein is expressed as [3H]/[35S] in the spot divided by [3H]/[35S] in total protein (trichloroacetic acid precipitate). In addition, a correction for the amount of material missing from the early portion of the graph since E₂₃₀ < E₀ was less than or equal to zero.

rotary water bath, and cell growth was followed in a Zeiss spectrophotometer at 420 nm. Cell growth was followed for at least six generations of steady-state growth before any experiments were initiated. Temperature shifts were initiated by diluting the culture growing at the preshift temperature into a flask containing 1/2 volume of culture medium preincubated at the postshift temperature.

Peptide elongation rate measured by β-galactosidase and alpha fragment induction. One way to determine the peptide chain elongation rate is to measure the time it takes to make β-galactosidase enzyme after induction with isopropyl-β-D-thiogalactopyranoside (IPTG). The time when the first β-galactosidase molecule is made can be extrapolated from the graph, and since we know that β-galactosidase contains 1,024 amino acids, we can calculate the number of amino acid molecules in the completed protein is expressed as [3H]/[35S] in the spot divided by [3H]/[35S] in total protein (trichloroacetic acid precipitate). In addition, a correction for the amount of material missing from the early portion of the graph since E₂₃₀ < E₀ was less than or equal to zero.
is fairly constant while elongation rate continues to increase. Growth is 2.5% faster at 42°C than at 37°C, but elongation rate increases from 12.9 (±0.31) to 16.9 (±0.41) amino acids/s, or approximately 30%. This is surprising because the levels of rRNA and protein are constant in this range (12, 28), and thus we would expect growth to be faster at 42°C than at 37°C. At very high temperature (>44°C), when growth rate begins to decrease dramatically, elongation rate also begins to decrease (data not shown).

We have also measured β-galactosidase induction time at low temperature (<25°C). The results in Fig. 3 demonstrate that elongation rate decreases with decreasing temperature; however, the rate of decrease of elongation rate at low temperature was greater than expected from the rate in the normal range of temperatures. Also apparent from the results in Fig. 3 is that when normalized to growth rate at 37°C, elongation rate does not decrease at low temperature as much as growth rate. This is also true when the values are normalized to the 23°C values; there is approximately a 50% greater decrease in growth rate than in elongation rate. This finding indicates that elongation rate is not limiting for growth at low temperature.

One possible artifact in the β-galactosidase induction experiment is any contribution of the time it takes for induction and initiation of β-galactosidase. For example, IPTG could be taken up more quickly by cells at 42°C than at 37°C. Therefore, an additional approach was used. Alpha fragment is the N-terminal portion of β-galactosidase (approximately 60 amino acids) and can be assayed independently of β-galactosidase. Since production of alpha fragment requires the same induction and initiation events that full-length β-galactosidase requires, the time between the first appearance of alpha fragment and the first β-galactosidase molecule is the time required to translate the ~975 amino acids between them. This time should be insensitive to any changes in induction or initiation. Alpha fragment induction at 15, 37, and 42°C occurred so quickly that it was indistinguishable from instantaneous induction (data not shown) at these temperatures. Therefore, any difference between the time needed to induce and initiate β-galactosidase at these temperatures is too small to change significantly the translation rates obtained and the conclusions presented above.

**Peptide chain elongation rate as determined by labeling kinetics.** Since the β-galactosidase induction method measures the translation time of a single protein, it may not be representative of the majority of proteins. Therefore, the pulse-chase method (see Materials and Methods) was used to measure the completion time of proteins at 37 and 42°C. The results of these studies confirm that the polypeptide chain elongation rate is higher at the higher temperature, although there is little difference in growth rate (Fig. 4). The elongation rates of RNA polymerase beta subunit are 24 amino acids/s at 42°C and 19 amino acids/s at 37°C. The elongation rate from the β-galactosidase experiments was lower, but the difference between the two temperatures was the same (approximately 30%). For the pulse-chase experiments, we routinely examined five or six proteins (β-galactosidase, RNA polymerase beta subunit, DnaK, ValS, polynucleotide phosphorylase, and pyruvate dehydrogenase kinase).

![Graph](https://via.placeholder.com/150)

**FIG. 2.** (A) Measurements of peptide chain elongation rate by the β-galactosidase induction method. Peptide chain elongation rate (closed circles) was calculated from the β-galactosidase induction time at the indicated temperatures. Growth rate (open circles) was determined by optical density measurements done on the same cultures. The average of data from several experiments is plotted with the standard error indicated. Points with no error bars had less than 2% standard error. aa, amino acids. (B) Peptide chain elongation rate relative to growth rate at various temperatures. Elongation rate as determined by β-galactosidase induction time at each temperature is plotted versus the specific growth rate at the same temperature. The error bars represent 1 standard error. Points with no error bars had less than 2% standard error.

![Graph](https://via.placeholder.com/150)

**FIG. 3.** Peptide elongation rate at low temperature. Peptide chain elongation rate (closed circles) was calculated from the β-galactosidase induction time at the indicated temperatures. Growth rate (open circles) was determined by optical density measurements done on the same cultures. The average from several experiments is plotted. The maximum standard error for any elongation rate value was 7%, and the maximum error for the growth rate was 2%. aa, amino acids.
vate dehydrogenase), but since translation is so fast at 42°C, only the results of the largest protein examined, RNA polymerase beta subunit, were consistently usable. The other proteins gave results which either confirm the RNA polymerase results or were merely consistent with those results qualitatively. Thus, at high temperature, there appears to be an excess of protein synthetic capacity in that the cells growing at 37 and 42°C contain the same number of ribosomes and the growth rates are the same, but there is a 30% increase in polypeptide chain elongation rate at 42°C. One possibility is that a fraction of ribosomes are inactive. Similarly, pulse-chase experiments were used to confirm the β-galactosidase induction results at 15°C, demonstrating that elongation rate is not limiting for growth at 15°C (data not shown).

Ribosome concentration. Previous studies have shown that the levels of ribosomal protein (12) and rRNA (28) are constant both within the normal temperature range and at high temperature. To confirm that the levels of ribosomes are constant between 37 and 42°C in our standard E. coli strain and our medium, the ratio of RNA to protein was measured in cells growing at each temperature. This ratio has been shown to reflect accurately the levels of ribosomes in cells when used to measure the levels in cells growing in different media (e.g., reference 21). These measurements showed no significant difference in the RNA/protein ratio between cells growing at 42°C (0.98 ± 0.04 [standard error]; n = 12) and those growing at 37°C (1.00 ± 0.02; n = 14). Therefore, the measurements made previously are confirmed, and we can conclude that cells growing at 37 and 42°C contain the same amount of ribosomal material.

Is all of this ribosomal material functional? We found no significant differences in the polysome profiles obtained from cells grown at 37 or 42°C (Fig. 5A). Several repetitions of this experiment showed that the percentages of polysomes, 70S monosomes, and 30S and 50S subunits were the same at the two temperatures with 62% (±1%, standard deviation) of the radioactivity found in the form of polysomes. Therefore, there is not a great pool of nonfunctioning ribosomes at 42°C compared to 37°C. From these results, we conclude that the number of functioning ribosomes per cell mass is constant in cells grown at 37 and 42°C. In contrast, polysome profiles from cells grown at 15°C (Fig. 5B) show that 20% of the ribosomes are in the form of 30S and 50S subunits, whereas less than 7% are subunits at 37°C.

Temperature shifts. It is of interest now to ask what happens after a shift to higher temperature. We expect one of two results: either the polypeptide chain elongation rate increases immediately upon a shift to 42°C, presumably as a passive response to the increased temperature, or the cell needs to actively increase the elongation rate, a process which could require time.

We measured the elongation rate after a shift either from 28 to 42°C or from 37 to 42°C (data not shown) by using β-galactosidase induction and after a shift from 37 to 42°C by the pulse-chase method. Induction or labeling was started at var-
TABLE 1. Effect of temperature shifts on polypeptide chain elongation rate

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Avg elongation rate (amino acids/s) ± SE</th>
<th>No. of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>16.9 ± 0.41</td>
<td>10</td>
</tr>
<tr>
<td>37</td>
<td>12.9 ± 0.31</td>
<td>18</td>
</tr>
<tr>
<td>28–42</td>
<td>15.4 ± 0.43</td>
<td>10</td>
</tr>
</tbody>
</table>

* Calculated from the β-galactosidase induction time in cells growing in steady state at indicated temperatures or 15 s to 5 min after a temperature shift from 28 to 42°C.

The results presented have shown that within the normal temperature range, the rate of polypeptide chain elongation increases, allowing faster growth without the need for increased synthesis of ribosomes. Thus, these results fit with our current understanding of the control of protein synthesis in E. coli.

We have found that cells growing at low temperature contain a large pool of ribosomal subunits (Fig. 5B). It is quite unusual to find a circumstance where a wild-type cell (growing in steady state) has a large pool of nontranslating ribosomes. The obvious question is why doesn’t the cell regulate its level of rRNA and protein such that it simply meets its needs at this temperature as it does in the normal temperature range? Cells growing at low temperature seem to require a higher concentration of ribosomes for protein synthesis. Perhaps this requirement is necessary to overcome the block in protein initiation as has been postulated (3, 6, 13), or possibly cells maintain a pool of ribosomes to meet future demands (14). Under most other conditions, E. coli cells maintain only the level of ribosomes per unit of cell mass required for protein synthesis. This seems sensible, since it would be very wasteful to maintain an excess of ribosomes. These extra ribosomes by definition do not contribute to cell growth. There are at least two situations in which it may make sense to have excess ribosomes. The first is in starvation or very slow growth. It is known that the relationship between the number of ribosomes per unit of mass and growth rate breaks down at very low growth rates (21). One reasonable model to explain this is that cells are “waiting for better days” (14). Slowly growing or starving cells which maintain a relatively high level of ribosomes would be able to respond rapidly to an increase in the nutritional richness of their medium. Applying the same logic to cells growing at low temperature, it is possible that these cells too are waiting for better days, i.e., a higher growth temperature.

When one considers our measurements of the fraction of functioning ribosomes (polysomes) at low temperature, our measurements of protein synthesis fit quite well with the observed growth rate. At 13.5°C, the levels of ribosomal proteins decrease to approximately 65% of the level at 37°C (12). The number of functional ribosomes (polysomes) is 83% of the 37°C value (Fig. 5B) and the peptide elongation rate is 11.6% of the rate at 37°C (Fig. 3). Using the 13.5°C value for the levels of ribosomal proteins (which should be close to the 15°C value), the protein synthetic capacity (number of ribosomes times the fraction working as polysomes times the elongation rate) at 15°C is 6.3% of the capacity at 37°C. This is quite similar to the difference in growth rate: cells at 15°C grow at 5.5% of the rate at 37°C.

At 42°C, where the specific growth rate is only about 2.5% higher than at that at 37°C, we have shown that the number of functioning ribosomes per unit of cell mass is approximately the same as at 37°C. Second, polypeptide chain elongation rate is not rate limiting for growth; it increases in proportion to temperature even at high temperature when growth rate is no longer increasing. RNA elongation rate is assumed to increase also, since in the β-galactosidase induction experiments the results are dependent on both translation and transcription rates. Also, the elongation rate of stable RNA has been shown to increase as a function of temperature and is proportional to growth rate (28). To summarize, the calculated protein synthetic capacity (defined above) at 42°C is 1.32 ± 0.05 (standard error calculated by using the Gaussian approximation formula) relative to the value at 37°C (1.0 ± 0.05). Thus, the results obtained at high temperature are surprising since they appear to violate our current models of the regulation of protein synthesis. It is possible that, as discussed above for cells grown at low temperature, cells growing at high temperature maintain a pool of ribosomes in anticipation of future demands. However, in this case we would expect to find a pool of nontranslating ribosomes in these cells (as at low temperature), which we do not. Thus, this explanation tells us nothing about the nature of the defect in cells growing at 42°C.

![FIG. 6. Peptide chain elongation rate after a shift to high temperature, determined by the pulse-labeling method. Cells growing at 37°C were shifted to 42°C, and the pulse was initiated 15 s later. Labeling was terminated after 15 s, and samples were taken for two-dimensional gel electrophoresis. Shown is the radioactivity isolated from a single protein spot (RNA polymerase beta subunit) versus time of chase. Radioactivity is expressed as described in Materials and Methods. The translation time is 55 s (indicated by the arrow), and the calculated elongation rate is 24 amino acids/s. The standard error of the calculated translation time is approximately 3%.](http://jb.asm.org/ Downloaded from)
A second model to explain why cells growing at high temperature appear to have an excess of protein synthetic activity involves protein degradation. It is possible that all of these ribosomes are indeed functioning and producing protein but that the rate of protein degradation is greatly increased. This model is very attractive in light of what we know about temperature upshifts and the heat shock response as discussed below. A problem with this model is that measurements of protein degradation at various temperatures have not shown an increase above the normal temperature effect (25, 26). It is possible, however, that degradation of newly synthesized protein can occur at rates high enough to account for the discrepancy. Most measurements of protein degradation have been done such that degradation only of long-lived proteins has been examined and show that 1 to 2% of cellular protein is degraded per generation (18). It is much more difficult to measure short-term degradation. Two studies have estimated the degradation of newly synthesized protein. First, Pine (26) showed that a minimum of 5% of the labeled amino acid incorporated in a 5-s pulse was released during the next 45 s. This rapid degradation was followed by a lower rate of about 2% per generation. A second series of experiments obtained similar results (37) showing that degradation of newly synthesized protein is significant and distinct from degradation of stable proteins. Furthermore, as seen in Fig. 4, we consistently find a decrease in the radioactivity in completed protein at 42°C, but not at lower temperatures, after the initial break in the curve, indicating that newly synthesized proteins are indeed degraded at a higher rate at high temperature than at normal temperatures. Thus, the rate of degradation of newly synthesized protein may be quite high (18). If, for some reason, polypeptides cannot fold as quickly at 42°C, newly synthesized protein would be more susceptible to proteolysis. We also show in this work that peptide elongation rate increases immediately upon an upshift in temperature. Temperature upshifts induce the heat shock response, which results in the induction of 20 or so proteins, many of which are chaperones or proteases (34; reviewed in references 11 and 22). The response is mediated by the induction of the sigma factor σ32. Do the observations about peptide elongation rate fit into our current thinking about the induction mechanism and function of the heat shock response?

The model of heat shock induction that results from the observations of σ32 induction and heat shock protein function can be summarized as follows (reviewed in reference 11). Upon an increase in temperature, the heat shock chaperones are increasingly bound to unfolded protein and are thus titrated away from σ32. This results in an increase in the stability of σ32 and its rate of synthesis (30, 33). The increase in σ32 levels is sufficient to induce the synthesis of the heat shock proteins, including the chaperones. When the levels of chaperones are sufficient to bind all of the cell’s unfolded protein, they can bind σ32 and decrease its level.

The model assumes that the concentration of unfolded protein is increased to induce a heat shock response. The source of unfolded protein probably differs in different inducing conditions, but a major source upon heat treatment is likely to be newly synthesized protein. As shown in this report, the rate of synthesis of nascent polypeptides increases immediately upon heat shock, and this may be the source of unfolded protein. To our knowledge there is no direct evidence that proteins synthesized before a temperature shift to 42°C actually unfold at the new temperature; thus, it seems likely that an imbalance between the rate of protein translation and the rate of growth triggers the heat shock response by causing a transient accumulation of newly translated, unfolded protein.

Implied in this model is that the rate of chaperone function does not increase in parallel to growth rate with increasing temperature or that the need for chaperone function increases faster than growth rate. Higher levels of the chaperones are required at higher temperatures. This could be for a number of reasons: the mechanism of chaperone function may not be a simple catalytic reaction, protein folding may be slowed by increasing temperature, or more proteins may require chaperones to help them fold at high temperature. A series of experiments by Horowitz and coworkers begins to address this issue (16, 17). They have studied the folding of rhodanese in vitro at different temperatures. Rhodanese will fold significantly at low temperature (10°C) without the presence of chaperones, but at a higher temperature (37°C), the protein precipitates. Addition of the chaperone GroEL/GroES enables the protein to fold at 37°C with no precipitation. Interestingly, addition of the chaperone at low temperature inhibits protein folding. This system is very similar to what is observed in vivo. Chaperone synthesis (and all heat shock protein synthesis) is inhibited at low temperature but is clearly required at high temperature. Heat shock mutants of either individual chaperones or σ32 form large protein-rich inclusion bodies at high temperature (reference 10 and unpublished results).

Thus, the increased rate of peptide chain elongation upon a heat shock fits this heat shock induction model quite well and in fact is a necessary part of the model. The increase in the rate of protein elongation is the trigger that generates the signal for the heat shock response.

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REFERENCES