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The Stress-Related Production of the Active *Photinus Pyralis* and *Luciola Mingrelica* Firefly Luciferases in *Escherichia Coli*

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ABSTRACT

The kinetics of *Photinus pyralis* and *Luciola mingrelica* luciferase gene expression was studied on plasmids with the thermoinducible λP_R promoter in *Escherichia coli* by SDS-gel electrophoresis of cell lysates to follow luciferase protein-synthesized, enzyme immunoassay (EIA) to follow native enzyme conformer, and the luciferase activity assay. *E. coli* cells were cultivated at temperature schemes 28–42–21°C or 28–21°C, or at alkali pH shift. In the cases of thermoinduction and pH shift, the luciferase expressions have similar features. The 3-h thermoinduction (42°C) followed by the incubation at 21°C, for 10 h resulted in the maximal amount of the luciferase protein of 4–5% of the total cell proteins. The yield did not change further. The amount of native luciferase conformer and the luciferase activity started to grow after incubation for 10 h at 21°C and reached the maximum after 50–60 h when the synthesized luciferase protein adopted the native-like conformation. At the same time, only 50% of the latter appeared to be catalytically active. An increase in the enzymatic activity correlates with an increase in the intracellular pH and ATP content. Intracellular metabolic reactions were shown to play a role in the conformational changes of the enzyme in a postthermoinduction period, and a possible mechanism of this effect is proposed.

Index Entries: *luc* gene; kinetics of gene expression; thermoinduction; firefly luciferase; *Escherichia coli*; plasmid; bioluminescence; pH shift.

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INTRODUCTION

Genetic engineering and biotechnology use a large number of *Escherichia coli* strains capable of synthesizing foreign proteins. The yields vary from 4–40% with respect to the total cell proteins (*1–5*). A firefly luciferase gene (*luc* gene) is an ideal gene marker (*6*) and a convenient model for the study of the kinetics of gene expression, since even a femtomolar quantity of luciferase in the cell lysate can easily be detected by the bioluminescent method. Large-scale biotechnological production of recombinant firefly luciferase is also increasingly important because of its numerous bioanalytical applications in industry and medicine (*7*). The influence of different external factors on yields and activity of recombinant proteins as well as the kinetics of their gene expression are not well understood. Earlier we investigated the so-called low-temperature induction phenomenon that occurred in the course of *Photinus pyralis* luciferase gene expression on the plasmid with thermoinducible λP_R promoter in *E. coli* (*8*). We showed that incubation of *E. coli* cells bearing this plasmid for 50 h at 21°C followed after 3 h of the thermoinduction at 42°C increased drastically (more than 100-fold) the luciferase activity in cells compared with the activity in cells right after the thermoinduction. In the other work, it was shown that not only the temperature, but also pH stress can significantly affect the yield of the active recombinant protein (*9*).

These findings raised the question whether the *P. pyralis* firefly luciferase gene expression is unique or the same is true for the *Luciola mingrelica* firefly luciferase gene. Both firefly luciferases are highly homologous (67% identity), but differ in some physicochemical properties (*10*). In this work, we studied in detail the kinetics of both *P. pyralis* and *L. mingrelica* firefly luciferase gene expression on the plasmids with the thermoinducible cI_{857}-regulated λP_R promoter in *E. coli* cells affected by the temperature or pH shifts. We looked at the factors that affect the amount and activity of recombinant luciferases, and at the mechanism of this "low-temperature induction."

MATERIALS AND METHODS

Strains, Media, and Plasmids

E. coli strain CA (Hfr H, thi-1, rel-1, lacI 22, lacZ 13, sup C70, cI857, χisl, RecA—) was used as a carrier for recombinant plasmids. *E. coli* cells were grown in LB broth containing 0.1 mg/mL ampicillin. The pME61 plasmid was constructed on a basis of the pMM40 plasmid and *P. pyralis* firefly luciferase gene kindly provided by D. Helinski (USA) (*8,11*). The pJGλ plasmid was constructed as shown in the Fig. 1 with pMM24 (*8*) and the *L. mingrelica* firefly luciferase gene derived from the pGK1 plasmid (*9*).

pJGλ :cro=ATG GAA CAA CGC ATA ACC TTG GGA TCT AGC AAA- luc
pME61:cro=ATG GAA CAA CGC ATA ACC TTG GGA TCC GGC CAA GC
TGG CAT TCC GGT ACT CTT GGT AAA-luc

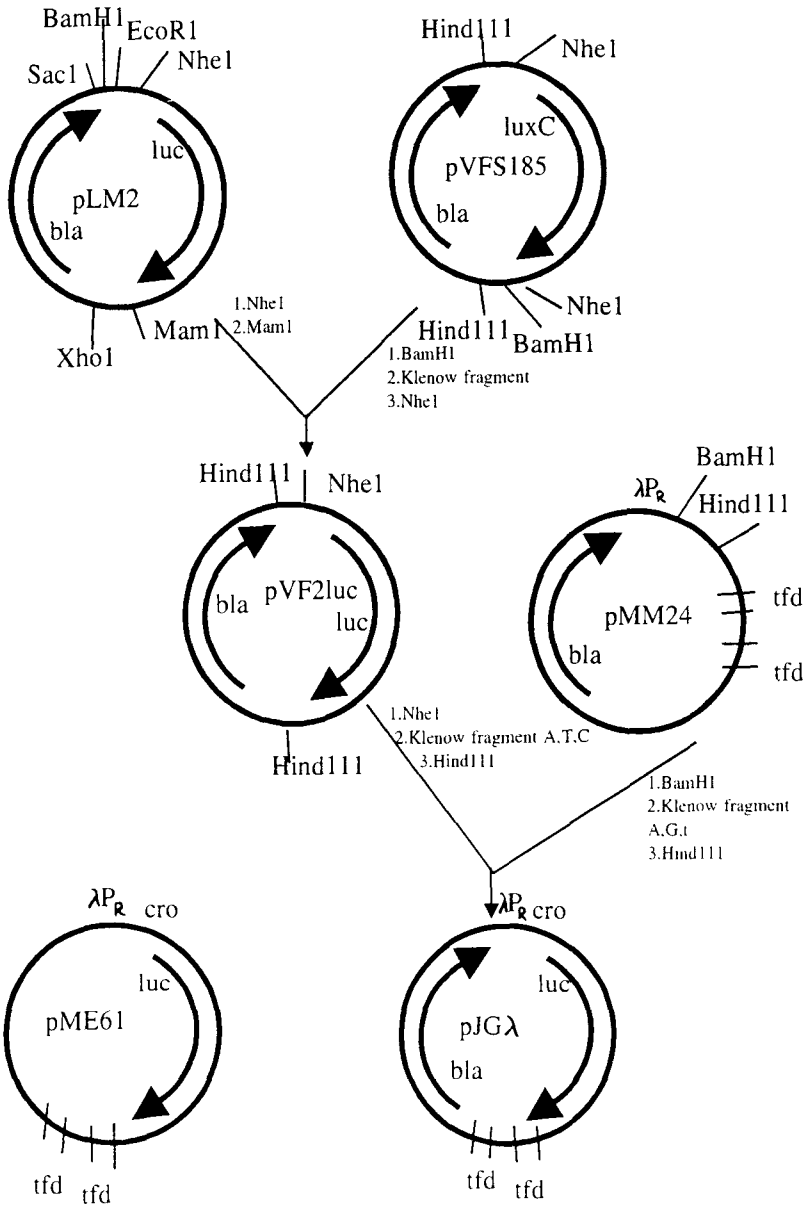

Fig. 1. The scheme of pJGλ synthesis and the structure of pME61.

Materials

Ampicillin, chloramphenicol, Triton-X-100, ATP, and SDS were obtained from Sigma. Bacto-tryptone and yeast extract were purchased from Merck. Luciferin was synthesized as described (*12*). The ATP reagent IMMOLUM was prepared as described (*7*). Polyclonal antibodies

against firefly luciferase and their conjugate with horseradish peroxidase were prepared at the Department of Chemical Enzymology, Moscow State University.

Kinetics of the *luc* Gene Expression

E. coli CA strains with recombinant plasmids were cultivated in 5 mL of LB broth at 28°C with shaking for 18 h, and then inoculated in 250 mL of LB broth and grown at 28°C until A_{590} of the culture reached 0.6. In the case of thermoinduction, the temperature was shifted to 42°C for 3 h, and then the culture was incubated at 21°C without shaking. In the case of the pH shift, the pH of the culture was increased to 8.5 or 9.0 by sterile concentrated Tris solution, and the cells were allowed to grow at 21°C with moderate shaking.

During the whole incubation period, 1.5 mL of the cell suspension aliquot was taken from the culture at certain time intervals. The cells were harvested by centrifugation and kept frozen at −20°C. For lysis, the cell pellets were resuspended at 0°C in 0.5 mL STET buffer, and then 0.05 mL of freshly prepared lysozyme solution (20 mg/mL) in 10 m*M* Tris-HCl, pH 8.0 were added to the lysate and the mixture was incubated at 0°C for 40 min. The cell debris in the lysate was precipitated by centrifugation for 5 min (14,000 rpm), and the supernatant was then analyzed.

Luciferase activity was assayed by the bioluminescent method using 0.6 mL 0.1*M* Tris-acetate buffer containing 10 m*M* $MgSO_4$, 2 m*M* EDTA, pH 7.8, 0.1 mL 4 m*M* ATP in the same buffer, 1 μL lysate, and 0.3 mL 4 m*M* luciferin. It was confirmed that the specific activity of the purified recombinant luciferase is the same as that of the native firefly luciferase (1.2×10^{17} arb.U/mol) (*13*). This activity was used for calculation of the amount of the active luciferase in lysate.

ATP concentration was determined using IMMOLUM reagent (*7*).

Luciferase protein in cell lysates was measured by the Laemmli SDS gel-electrophoresis (*14*). The total protein concentration in the cell lysates was determined by the Bradford method. The luciferase protein (mg/μL of lysate) was evaluated by densitometry of luciferase band on the SDS gels with dual-wavelength flying-spot scanner "Shimadzu CS-9000."

Enzyme immunoassay was carried out by the standard "sandwich method" using polyclonal antiluciferase antibodies and a conjugate of antibodies with horseradish peroxidase.

Intracellular pH was determined as described (*15*) with minor modifications. Two milliliters of cell suspension were precipitated by centrifugation. The cell pellet was consequently washed with LB medium, HEPES, and TES buffers, and then resuspended in a buffer with pH from 5.0–9.0. 9-Aminoacridine was added to the cell suspension. Its final concentration was 2 mg/mL, and the quenching of fluorescence was measured as a function of pH.

RESULTS

The kinetics of the *P. pyrallis* and *L. mingrelica* firefly luciferases gene expression was studied in *E. coli* cells, CA strain (*see* Materials and Methods) carrying pME61 or pJGλ plasmids (Fig. 1). The cell cultures were grown at 28°C until they reached A_{590} = 0.6, and then the thermosensitive cI_{857} repressor was inactivated by raising the temperature to 42°C. The cell growth practically stopped after 3 h of thermoinduction. During the whole period of incubation, aliquots of cell suspension were withdrawn and cell lysates were prepared as described in Materials and Methods. The luciferase content in cell lysates was analyzed by:

1. The bioluminescent assay of the luciferase activity;
2. The SDS gel electrophoresis to estimate the total amount of luciferase protein; and
3. The enzyme immunoassay (EIA) to calculate the quantity of the luciferase native conformer.

Time-Course of Recombinant Luciferase Activity During *luc* Gene Expression

During the first 3 h of thermoinduction, the activity of the recombinant enzyme increased from 10–60 arb. U/1 μL lysate for *L. mingrelica* luciferase and 120 arb. U/1 μL lysate for *P. pyralis* luciferase. Further incubation of cells at 42°C up to 20 h decreased the activity to zero probably because of thermoinactivation of the synthesized enzyme, which is known to be labile (*16*). It was shown on studying the expression of rice lipoxygenase L gene in *E. coli* (*17*) that the enzyme synthesized at 37°C was totally inactive, but if the synthesis was performed at 15°C, the enzyme was active. The thermoinactivation of synthesized recombinant protein during thermoinduction (42°C) was observed also for other expressed proteins as well (*18*).

In our previous work (*8*), we described a so-called low-temperature induction of the enzyme activity for the expression of *P. pyralis* firefly luciferase gene in *E. coli*. If *E. coli* cells carrying the plasmid with a thermoinducible promoter and *P. pyralis* firefly luciferase gene were incubated at 21°C without shaking right after 3 h of thermoinduction, the cells did not breed any more, but the activity of luciferase inside the cells (determined in the cell lysates) was steadily increasing. It reached the maximum (ca. 100-fold increase in activity) on incubation for 50–60 h at 21°C, remained at this level (1.8×10^4 arb. U/1 μL lysate) for the next 50 h, and then slowly decreased (Fig. 2, curve 1).

The same experiments were carried out for the expression of *L. mingrelica* firefly luciferase gene in *E. coli*. An increase in the activity of *L. mingrelica* luciferase in *E. coli* cells on incubation at 21°C appeared to be similar to that of *P. lyralis* luciferase, though the maximal activity of the former

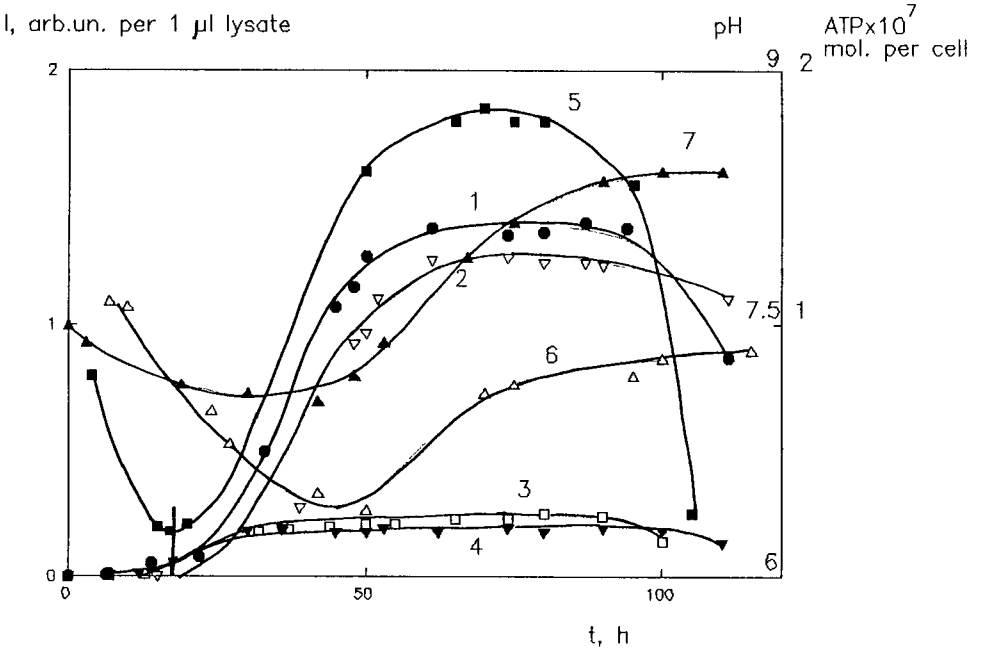

Fig. 2. Time-courses of the firefly luciferase activity in lysates of *E. coli* cells carrying pME61 (1, 3) or pJGλ (2, 4), intracellular ATP content (5), pH_{in} (6) and pH_{out} (7). The cells were incubated according to the temperature scheme 28–42–21°C in the absence (1, 2) and in the presence (3, 4) of chloramphenicol. The arrow indicates a moment of chloramphenicol addition. For conditions, *see text*.

was slightly lower (1.2×10^4 arb. U/1 μL lysate) (Fig. 2, curve 2). Thus, the low-temperature induction is a general phenomenon.

To check the importance of thermoinduction for the active luciferase synthesis during the culture incubation at 21°C, we carried out the control experiments without thermoinduction. The *E. coli* cells with plasmids bearing *luc* gene were grown at 28°C for 8 h until they reached $A_{590} = 1.6$ and then incubated at 21°C without shaking. The luciferase activity increased only two- to threefold (Fig. 3) during the incubation at 21°C.

We checked the viability of *E. coli* cells after the long-term expression (thermoinduction and subsequent incubation at 21°C) and found that 70% of the cells were viable. The cell suspension with the highest luciferase activity (50–60 h of expression) was used to start a new cell culture in a whole expression cycle using the temperature scheme 28–42–21°C. During the second expression cycle, it took a longer time for the cells to reach $A_{590} = 0.6$, but the maximal luciferase activity was the same as in the first round (Table 1).

The Kinetics of Firefly Luciferase Protein Accumulation for *luc* Gene Expression in *E. coli*

The sharp increase in the luciferase activity in *E. coli* cells in the post-thermoinduction period (Fig. 2) could be explained both by an increase in

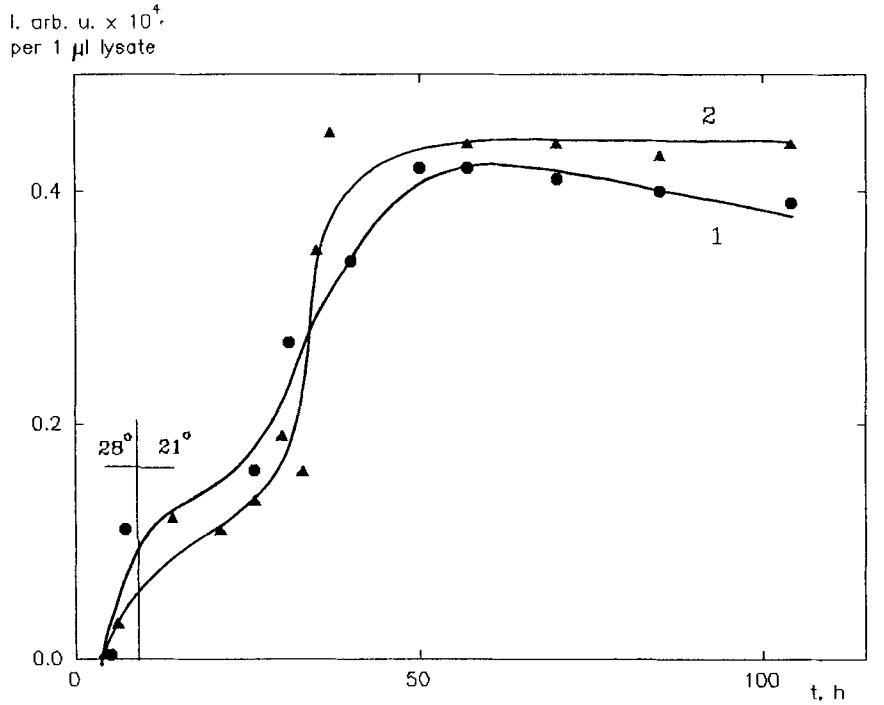

Fig. 3. Time-course of the *P. pyralis* (1) and *L. mingrelica* (2) luciferase activity in *E. coli* cell lysates. The cells were incubated according to the temperature scheme 28–21°C without thermoinduction. Other conditions are as in Fig. 2.

Table 1
Activity of Recombinant Luciferase
During the Cyclic Growth of the Cells

Number of cycles	Maximum activity of *P. pyralis* luciferase, arb. U	Time required for *E. coli* cells to grow until A_{590} = 0.6 h
0	1.4×10^4	3.7
1	1.3×10^4	4.8
2	1.3×10^4	4.9
3	1.5×10^4	4.6

For a "0 cycle" a fresh cell culture was used. For the next cycles, the cell culture after 3 h of thermoinduction (42°C) followed by the 50-h incubation at 21°C was used.

the catalytic activity of the synthesized protein and/or by the protein synthesis during 21°C incubation period. The SDS gels of *E. coli* cell lysates without *luc* gene had no band corresponding to a protein with molecular weight of firefly luciferase (61 kDa) (Fig. 4).

In *E. coli* cells carrying the *luc* gene plasmids, the luciferase protein appeared after 3 h of thermoinduction, then grew three times in 10 h during the 21°C incubation, reached 4.1 and 4.5% of the total cell protein for

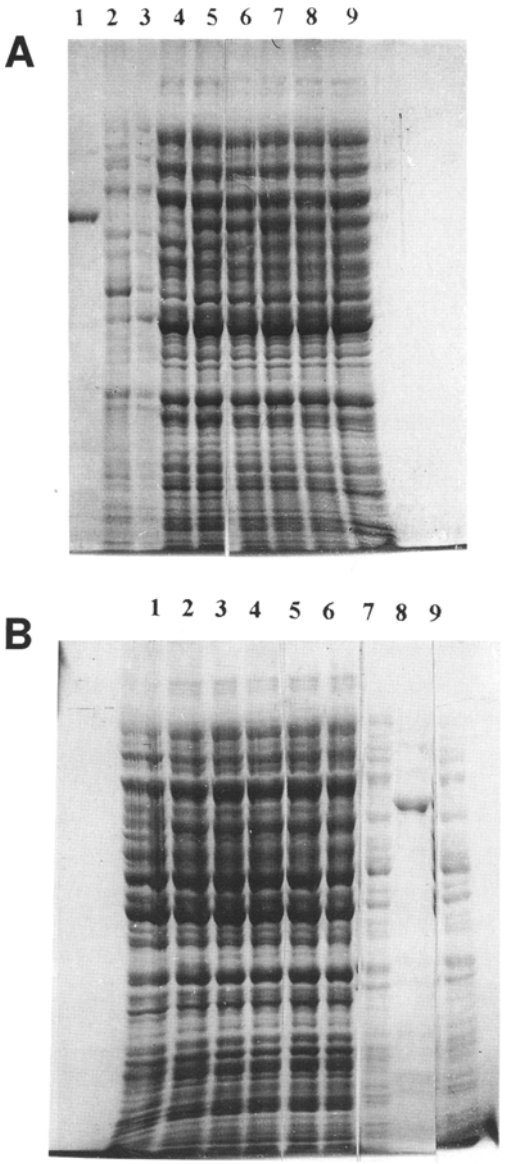

Fig. 4. SDS-PAGE of lysates of *E. coli* cells synthesizing *L. mingrelica* (**A**) and *P. pyralis* (**B**) luciferases under conditions of 3 h of thermoinduction. A: Purified luciferase (1); lysate of cells without *luc* gene (2); cell lysate after cell incubation for, h: 3 (3), 7 (4), 12 (5), 20 (6), 45 (7), 70 (8), 100 (9). B: Cell lysate after incubation for, h: 100 (1), 70 (2), 45 (3), 30 (4), 20 (5), 12 (6), 3 (7), purified luciferase (8), lysate of cells without *luc* gene (9).

L. mingrelica and *P. pyralis* luciferases, respectively, and did not change further (Fig. 4). At the same time, the luciferase activity increased by a factor of 120–200 during the incubation. In the absence of thermoinduction, the growth of both culture and luciferase protein stopped simultaneously reaching 2.4 and 2.8% of the total cell protein for *L. mingrelica* and

P. pyralis luciferases, respectively. Thus, an increase in the luciferase activity in the postthermoinduction period was probably not only owing to *de novo* synthesis of the luciferase protein, but mainly to transformation of luciferase from inactive to the catalytically active form.

The Kinetics of Transformation of Recombinant Luciferase into the Native-like Conformer According to EIA Data

The increase in luciferase activity could arise from:

1. A posttranslational modification of the synthesized luciferase;
2. Refolding of the thermally denaturated enzyme into the active conformation, and
3. Binding of the synthesized luciferase with activators or removal of inhibitors.

We applied EIA to find out to what extent the increase in activity could be owing to the refolding. In preliminary experiments in vitro, we checked that both *L. mingrelica* and *P. pyralis* thermoinactivated luciferases did not bind to polyclonal antibodies raised against luciferase. It is well known that native firefly luciferases have similar antigenic determinants so they crossreact with each other (*19*). Using the polyclonal antibodies against *L. mingrelica* luciferase, we followed the time-course of accumulation of the native conformer for both luciferases (Fig. 5A, B) that appeared to correlate with the time-course of an increase in the luciferase activity. The amount of the active luciferase conformer reached almost 100% of the total luciferase protein. However, only 50% was catalitically active (calculated using the specific activity for luciferase; *see* Materials and Methods).

These data clearly demonstrate that even though all thermoinactivated luciferase refolded into the native-like conformation in the postthermoinduction period, the luciferase activity did not recover in full. The question still remained open. Luciferase with native-like conformation could be half as active as the native luciferase, or there could be two luciferase with native-like conformations, one of which is fully active and the other totally inactive. The experiments with both *L. mingrelica* and *P. pyralis* recombinant luciferases (*10*) showed that purified recombinant luciferases have the same specific activity as the native firefly luciferase purified. This supports the existence of the two native-like luciferase conformations. Probably, the transformation of the native-like luciferase conformer into the active one could be very slow and not completed within the postthermoinduction period.

The Role of Intracellular Processes in Synthesis and Activation of Firefly Luciferases

To check whether the luciferase activity originated in vivo during the postthermoinduction period could also be achieved in cell lysate in vitro,

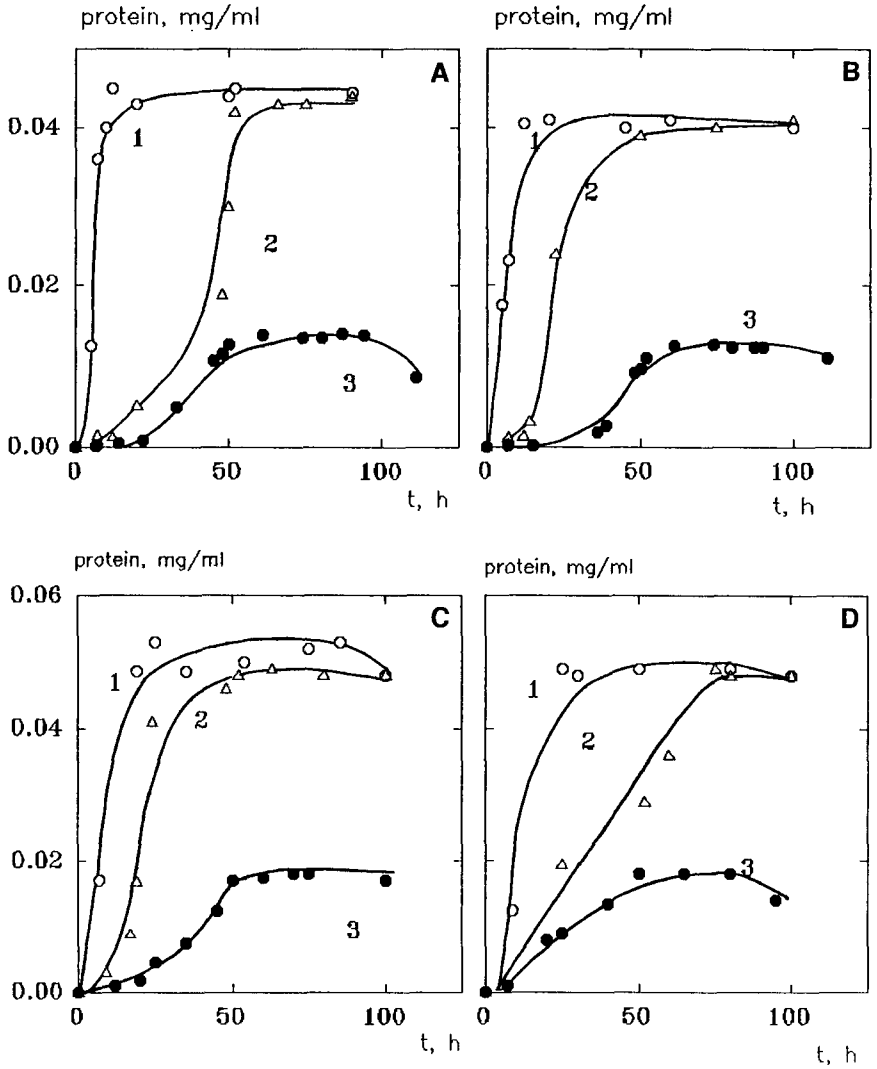

Fig. 5. Time-courses of *L. mingrelica* (**A** and **C**) and *P. pyralis* (**B** and **D**) firefly luciferases according to SDS electrophoresis (1), enzyme immunoassay (2), and the activity of luciferase in cell lysates (3). The expression of *luc* gene was induced by the temperature (A and B) and pH shift to 9.0 (C and D).

the lysates prepared from the cells right after thermoinduction were incubated at 21°C in the presence of glutathione, ditiotheitol, and urea. The luciferase activity did not change on incubation at 21°C, suggesting that some intracellular processes may be involved in the transformation of the thermoinactivated luciferase into the active form. We found a correlation between the activity of luciferase and the amount of intracellular ATP (Fig. 2). The period of maximal content of ATP (1.8×10^7 molecules/cell) coincided with that of luciferase activity. When the inhibitor of protein biosynthesis, chloramphenicol, was added to the culture at a moment

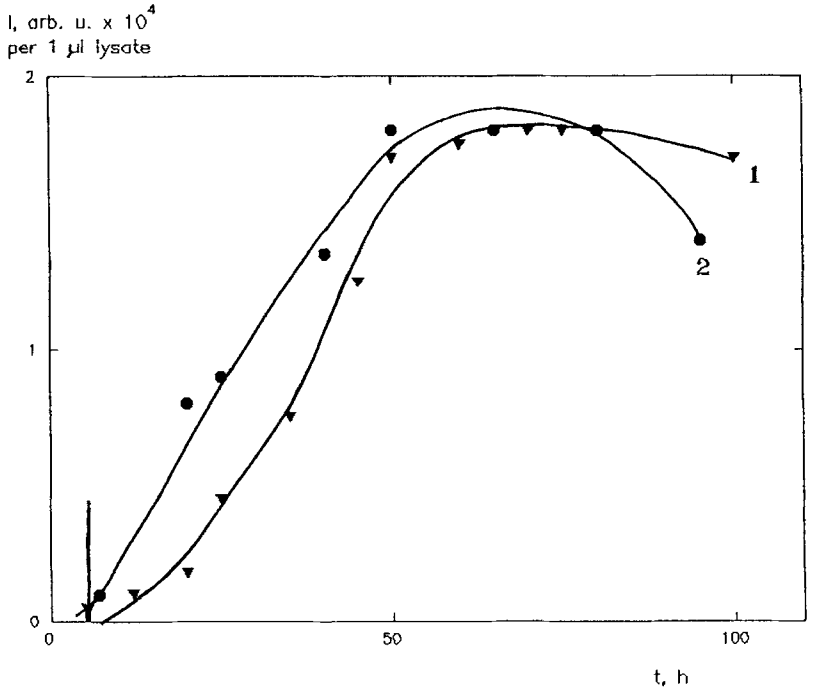

Fig. 6. Time-course of the *L. mingrelica* (1) and *P. pyralis* (2) luciferase activity in *E. coli* cells after pH shift. The arrow indicates a moment of pH shift.

when the maximal amount of luciferase protein was synthesized, we observed neither luciferase activity nor intracellular ATP increase. These data certify that cell metabolism is intensified in the late poststationary phase, although the reasons are not clear yet.

The observed activation of luciferase in vivo could be owing to changing physiological parameters inside a host cell, in particular, of intracellular pH. We measured pH in the culture medium (pH_{out}) in parallel with the assay of luciferase activity (Fig. 2, curve 6). After 3 h of thermoinduction during the incubation of *E. coli* culture at 21°C, the pH_{out} decreased first from 7.50 to 7.10–7.15 and then increased to 8.3–8.5. The growth of luciferase activity in the cells correlated with an increase in pH_{out}.

We further checked if biosynthesis and activation of luciferases could occur without thermoinduction but because of an increase of pH_{out}. The cell culture was incubated at 28°C until A_{590} reached 0.6, and then we shifted the pH_{out} to 9.0. After 30–40 h of incubation at 21°C, we observed a sharp increase in luciferase activity up to 1.8×10^4 arb. U/1 μL lysate (Fig. 6). When pH_{out} was shifted to 8.5, the increase was also observed with the maxima of 1.7×10^4 and 1.2×10^4 arb. U/1 μL lysate for *P. pyralis* and *L. mingrelica* luciferases, respectively. The gel electrophoresis of *E. coli* cell lysates (Fig. 7) demonstrated that the amount of luciferase protein in cells reached the maximum (4.5–5.3% of total cell proteins) within 17 h after the pH shift and did not change further (Fig. 5C and D, curve 1).

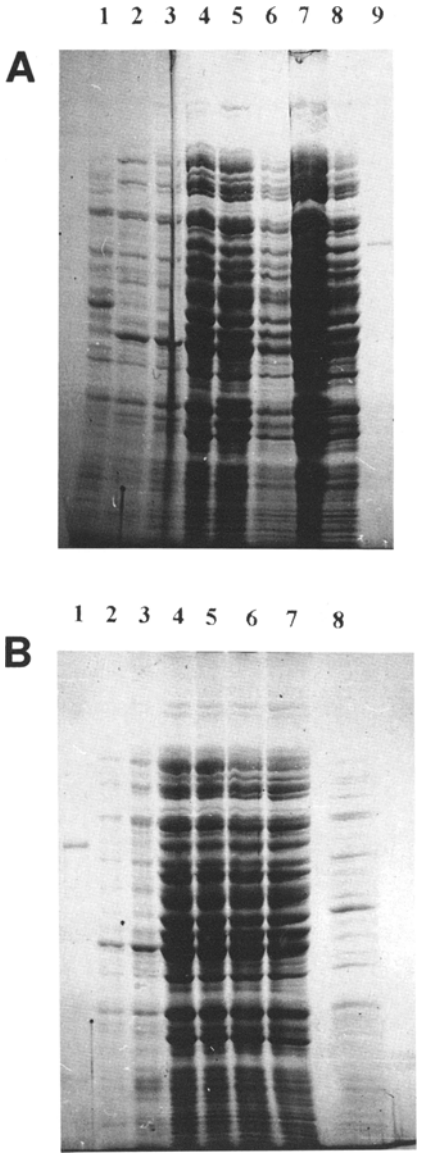

Fig. 7. SDS-PAGE of lysates of *E. coli* cells synthesizing firefly *L. mingrelica* (**A**) and *P. pyralis* (**B**) luciferases under the pH shift to 9.0. A: Lysate of cells without *luc* gene (1); cell lysate after incubation for, h: 3 (2), 9 (3), 24 (4), 45 (5), 65 (6), 80 (7), 100 (8), purified luciferase (9). B: Purified luciferase (1), cell lysate after incubation for, h: 3 (2), 9 (3), 25 (4), 50 (5), 80 (6), 100 (7), lysate of cells without *luc* gene (8).

Hence, the pH stress as the temperature stress induced the synthesis of recombinant protein and enzyme activation. For both luciferases, an increase in the luciferase activity in the cells after the pH_{out} shift corrrelated also with the accumulation of the native conformer (by EIA analysis; Fig. 5). In both cases, the maximum content of the native-like luciferase con-

former reached 100% with respect to the total amount of luciferase synthesized. The content of the active luciferase was, however, not more than 30%.

The intracellular pH (pH_{in}) in *E. coli* cells is known to depend significantly on the conditions of cell cultivation (*20–22*). Since the activation of luciferases in the *E. coli* cells results from a change in pH of the culture medium (pH_{out}), it seems very likely that changing in intracellular pH (pH_{in}) that should be dependent on the pH_{out} plays a major role in this process. Our results demonstrated that pH_{in} decreased from 7.6–6.3 within first 50 h of incubation (whereas pH_{out} decreased to 7.1) and then increased to 7.3 (Fig. 2, curve 7). This pH_{in} increase correlated well with the growth of the luciferase activity. Hence, the activation of protein synthesized began simultaneously with the alkalization of the intracellular medium.

DISCUSSION

The kinetic study of *luc* gene expression in *E. coli* showed different time-courses for the biosynthesis of recombinant luciferases and their transformation into native conformation. Luciferase protein biosynthesis is completed after 10 h of incubation at 21°C followed thermoinduction (42°C). It results in the formation of practically inactive, although soluble enzyme. Its folding into native conformation has a large induction period (up to 40–50 h) and is a result of the activation of intracellular metabolism and pH_{in} changes. The amount of active luciferase reaches only 50% of synthesized protein (or native-like conformer).

It seems possible that luciferase synthesized during the cell thermoinduction at 42°C undergoes a slow transformation into the native conformation and then into a catalytically active form in the postthermoinduction period at 21°C. The intracellular processes are activated during the postthermoinduction period as is evident from an increase in intracellular ATP along with a change in intracellular pH. The folding of inactive luciferase is slow and does not give full recovery of the activity though all protein obtains finally native-like conformation. The luciferase activation after alkali pH_{out} shift could be explained in the same way, though the stress conditions were not eliminated in this case. This is probably why the level of active luciferase recovery was higher in thermoinduction than in the alkali pH_{out} shift.

In conclusion, we would like to emphasize that while studying the kinetics of foreign protein gene expression on plasmids with thermoinducible promoters, it is important to take into account the fact that the level of the expressed protein marker calculated from its enzymatic activity does not always correspond to the total amount of the synthesized protein. The biosynthesis and transformation of protein into the active conformation might have different time-courses. The catalytically active enzyme, as we have shown for both firefly luciferases in *E. coli* cells, appears

with a significant delay (to 20–40 h) compared with its biosynthesis. The processes that occur inside a host cell under stress conditions, i.e., accumulation of ATP, the change in intracellular pH, and so on, play an important role in the folding and activation of recombinant protein.

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