# Role of Hsp70 (DnaK−DnaJ−GrpE) and Hsp100 (ClpA and ClpB) Chaperones in Refolding and Increased Thermal Stability of Bacterial Luciferases in *Escherichia coli* Cells

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**Abstract**—The role of chaperones Hsp70 (DnaK−DnaJ−GrpE) and Hsp100 (ClpA−ClpB−ClpX) in refolding of thermoin-activated luciferase from the marine bacterium *Photobacterium fischeri* and the terrestrial bacterium *Photorhabdus luminescens* has been studied. These luciferases are homologous, but differ greatly in the rate of thermal inactivation and the rate constant for the luminescence reaction. It was shown that refolding of thermoinactivated luciferases is completely determined by the DnaK−DnaJ−GrpE system. However these luciferases markedly differ in the rate and degree of refolding. The degree of refolding of thermolabile "quick" *Ph. fischeri* luciferase reaches 80% of the initial level over several minutes, whereas renaturation of thermostable "slow" *Ph. luminescens* luciferase proceeds substantially slower (the degree of renaturation reaches only ~7-8% of the initial level over tens of minutes). The measurement of the rate of thermal inactivation of luciferases *in vivo* in the cells of *Escherichia coli* wild strain and strains containing mutations in genes *clp*A, *clp*B, *clp*X showed that *Ph. luminescens* luciferase revealed reduced thermostability in mutant strain *E. coli clp*A$^-$. It was shown that this effect was not connected with DnaK-dependent refolding. In the case of thermolabile *Ph. fischeri* luciferase, mutation in gene *clp*A has no effect on the shape of the curve of thermal inactivation. These data suggest that denatured *Ph. luminescens* luciferase has enhanced affinity with respect to chaperone ClpA in comparison with DnaK, whereas thermolabile *Ph. fischeri* luciferase is characterized by enhanced affinity with respect to chaperone DnaK. Denatured luciferase bound to ClpA does not aggregate and following refolding proceeds probably spontaneously and very quickly (over 1-2 min). It is evident that the process under discussion requires ATP, since the addition of uncoupler of oxidative phosphorylation carbonyl cyanide 3-chlorophenylhydrazone results in a sharp decrease in thermal stability of luciferase to the level typical of the enzyme *in vitro*. The enhanced thermosensitivity of luciferases was observed also in *E. coli* containing mutations in gene *clp*B. However, this effect, which takes place for *Ph. fischeri* luciferase as well as for *Ph. luminescens* luciferase, is determined by DnaK-dependent refolding and probably connected with the ability of chaperone ClpB to provide disaggregation of the proteins, resulting in their interaction with chaperones of the Hsp70 family (DnaK−DnaJ−GrpE).

*Key words*: chaperones, DnaK, ClpA, ClpB, refolding, luciferase

In a bacterial cell molecular chaperones participate in many processes connected with correct folding of nascent polypeptide chains during and after translation, with recovery of the native structure of partially denatured proteins (refolding), with disaggregation of protein supramolecular structures formed in the cell during heat shock, with translocation through membranes, and so on [1-5]. Firefly luciferase is widely used as a model protein for study of the role of various chaperone systems in fold-

ing and refolding of proteins [6-8]. In particular, it was shown that refolding of denatured firefly luciferase requires a combined action of chaperones belonging to the Hsp70 family [8, 9].

In the present paper the influence of bacterial chaperones of Hsp70 family (DnaK−DnaJ−GrpE) and Hsp100 family (ClpA, ClpB, ClpX) on thermal stability and refolding of bacterial luciferases has been studied. Bacterial luciferases are among the group of thermolabile proteins, which undergo inactivation at 37-42°C. This circumstance allows the peculiarities of thermal

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inactivation and refolding of these enzymes immediately in a bacterial cell to be studied. In contrast to monomeric firefly luciferase, bacterial luciferases are heterodimers consisting of $\alpha$- and $\beta$-subunits with molecular masses of ~40 and 35 kD, respectively. Subunits of luciferases are homologous. The active site of the enzyme is located on the $\alpha$-subunit. The role of the $\beta$-subunit remains unclear, but its presence in the complex enhances greatly the quantum yield of the reaction [10, 11]. In the present paper thermolabile luciferase from the marine bacterium *Photobacterium fischeri* and thermostable luciferase from the bacterium *Photorhabdus luminescens* were used. Earlier we showed that DnaK-dependent refolding is more efficient in the case of thermolabile luciferase from *Ph. fischeri* [12]. In the present work we have studied the role of chaperones ClpA and ClpB in refolding of *Ph. fischeri* and *Ph. luminescens* luciferases as well as the influence of these chaperones on thermal stability of luciferases in the cells of *Escherichia coli*. ClpA and ClpB ATPases belong to the family of chaperones Hsp100 and play an important role in unfolding and proteolysis of some bacterial proteins [13]. It was shown also that chaperone ClpB participates in refolding of proteins, providing disaggregation of denatured proteins *in vitro* as well as *in vivo* under the action of Hsp70 chaperones (DnaK−DnaJ−GrpE) [14-16]. As for ClpA, it was shown *in vitro* that this chaperone forms a complex with denatured polypeptides irrespective of whether or not these proteins are the substrates of ClpAP-proteinase. However ClpA does not participate in DnaK-dependent refolding of proteins [17, 18].
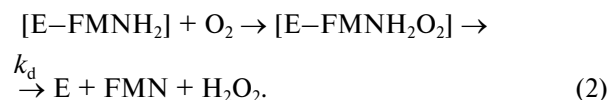
## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *E. coli* K12 SG20250 $\Delta lac$U169 *ara*D *flb*B *rel*A *clp*A$^+$ *clp*B$^+$ and its insertion mutants SG22099 *clp*A::kan *clp*B$^+$ and SG22100 *clp*A$^+$ *clp*B::kan were obtained from S. Gottesman and Y. Zhou (USA) [19]. *E. coli* K12 MG1655 and its deletion mutant PK202 $\Delta dna$K14 $\Delta dna$J14 *dks*A::kan were obtained from E. Craig (USA) [20]. *E. coli* K12 AB1157 and its deletion mutants NK113 $\Delta clp$P::cat and NK114 $\Delta clp$X::kan were obtained from N. E. Murray [21]. *E. coli* K12 TG1 was obtained from the collection of GosNIIgenetika.

Hybrid plasmid pF2 with genes *lux*AB *Ph. fischeri* placed under promotor $P_{lac}$ was constructed by recloning of DNA fragment from plasmid pF1 containing full *lux*-operon of *Ph. fischeri* [22]. Hybrid plasmid pXen4 contains fragment of DNA with genes *lux*AB recloned from hybrid plasmid pXen7 with full *lux*-operon of *Ph. luminescens* Zm1 [23].
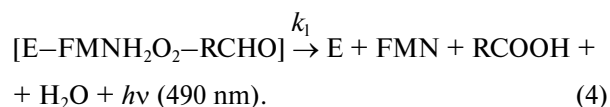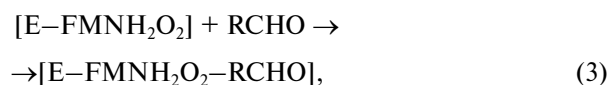
**Isolation and purification of bacterial luciferases.** Bacterial cells containing hybrid plasmids with genes *lux*AB were grown in L-broth with ampicillin (100 μg/ml)

at 28°C on a shaker till absorbance reached 2.5-3.0. After centrifugation the cells were resuspended in a tenth of the solution volume containing 50 mM phosphate buffer, pH 7.0, and 20 mM $\beta$-mercaptoethanol. Protein extract was obtained by treatment of the suspension of the bacterial cells (portion-wise in 30 ml) by ultrasound (44 kHz; 6 times for 20 sec each time) with cooling in ice. The sonicated suspension was centrifuged for 30 min at 30,000*g* to remove cell fragments. Further purification was achieved by passing the protein through the column with Sephadex G-25; 0.1 M potassium phosphate buffer, pH 7.0, was used for elution. To detect the fractions containing luciferase, the bioluminescence method was applied. The content of protein in a tube after purification on the column with Sephadex G-25 was ~100 μg/ml.

**Measurement of the intensity of bioluminescence and the rate constant of the enzymatic reaction.** Bacterial luciferases (E) catalyze oxidation of long-chain aldehyde (RCHO) by oxygen ($O_2$) of air with the participation of reduced flavin mononucleotide ($FMNH_2$): $FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O$ + a quantum of light (490 nm). The reaction proceeds through several stages:

$$E + FMNH_2 \rightarrow [E-FMNH_2], \tag{1}$$

$$[E-FMNH_2] + O_2 \rightarrow [E-FMNH_2O_2] \rightarrow$$
$$\overset{k_d}{\rightarrow} E + FMN + H_2O_2. \tag{2}$$

Complex $[E-FMNH_2O_2]$ breaks down through the dark pathway (2) with the rate constant $k_d$ or interacts with aliphatic aldehyde with the formation of ternary complex (3) which breaks down with generation of a quantum of light ($k_1$ is the rate constant of this reaction) (4):

$$[E-FMNH_2O_2] + RCHO \rightarrow$$
$$\rightarrow [E-FMNH_2O_2-RCHO], \tag{3}$$
$$[E-FMNH_2O_2-RCHO] \overset{k_1}{\rightarrow} E + FMN + RCOOH +$$
$$+ H_2O + h\nu \ (490 \ nm). \tag{4}$$

The fact that the reaction is accompanied by generation of a quantum of blue-green light (490 nm) provides a possibility of simple and quick measuring the enzymatic activity of luciferase in the model systems *in vitro* as well as *in vivo* without destroying the bacterial cell.

The rate constant of luminescence decay ($k_t$) is determined by the following expression: $k_t = (k_1A + k_dK_a)/(K_a + A)$, where $K_a$ is the dissociation constant for the ternary complex and $A$ is the concentration of aliphatic aldehyde [24]. We used high concentrations of aliphatic aldehyde *n*-decanal ($A \gg K_a$), where the following relationship was valid: $k_t \simeq k_1$.

Bioluminescence of the bacterial cells and cell-free extracts was measured with the aid of a model 1251 luminometer (Finland) at high concentration of *n*-decanal (6.8 µM) at 20°C. The reaction mixture in the *in vitro* measurements contained 0.1 M potassium phosphate buffer, pH 7.0, 20 mM β-mercaptoethanol, 20 µM FMN (Sigma, USA), and 6.8 µM *n*-decanal (Sigma). The reaction was initiated by the addition of 20 µl of 0.1% solution of sodium dithionite (Fisher, Germany), which reduces FMN to $FMNH_2$, to the cell with the reaction mixture placed immediately before the photomultiplier [25]. The final volume of the reaction mixture was 1 ml. Oxidation of $FMNH_2$ proceeds quickly in comparison with the life time of intermediates of the luminescence reaction. Therefore, the enzyme exerts only one turnover, the intensity of emission reaching a maximum for the first several seconds. Further decrease in the reaction rate and the intensity of luminescence (*I*) proportional to the reaction rate is described by the first order kinetics: $I = I_0 \exp(-kt)$ [26]. The rate constant of the luminescence quenching ($k = k_1$) was determined by calculating the slope of the straight line in coordinates {ln*I*; *t*} (*t* is time). In this procedure we used only part of the decay curve corresponding to the interval of the $I/I_{max}$ values from 0.8 to 0.2 ($I_{max}$ is the maximum value of the intensity of luminescence).

**Thermal inactivation and refolding of luciferases.** Thermal inactivation of luciferases *in vivo* and *in vitro* was carried out in a water bath at a fixed temperature. In the experiments *in vivo* chloramphenicol (167 µg/ml) was added to the suspension of the cells to suppress the protein synthesis. To decrease ATP concentration in bacteria, we used uncoupler of oxidative phosphorylation carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma) as well as FCCP. ATP concentration in the bacterial cells was measured using firefly luciferase [27]. Refolding of luciferases was carried out at room temperature or at a temperature that is optimum for the given luciferase: 25°C for *Ph. fischeri* luciferase and 35°C for *Ph. luminescens* luciferase. Bioluminescence was measured in aliquots (200 µl) withdrawn at certain intervals. Preliminary "heat shock" was carried out by incubation of *E. coli* cells in the thermostat at 42°C in the absence of chloramphenicol.

## RESULTS AND DISCUSSION

**Thermal stability and rate constant for the luciferase reaction.** Luciferases of *Ph. fischeri* and *Ph. luminescens* are highly homologous: 66% of the total number of the amino acid residues in the α-subunit (359 residues) and 52% of the total number of amino acid residues in β-subunit (324 residues) are identical [28]. However, these luciferases differ greatly in the rate of thermal inactivation and the rate constant for the luciferase reaction. Figure 1

shows the kinetic curves of thermal inactivation of the purified preparations of luciferases from *Ph. fischeri* and *Ph. luminescens* at 43.5°C. The comparison of the slopes of the kinetic curves allows the following conclusion to be made: *Ph. luminescens* luciferase loses the activity ~15 times slower than *Ph. fischeri* luciferase. The values of the rate of the enzymatic reaction for these luciferases differ by a factor of ~10-15 (table). The table summarizes the average values of the rate constants for the luciferase reaction and the rate constants of thermal inactivation (averaging over the results of five experiments in each case). It should be noted that the above characteristics of the luciferases are determined by the α-subunit, where the active site is located [24].

**Refolding of thermoinactivated luciferases.** Registration of the kinetics and degree of refolding of thermoinactivated luciferases was carried out *in vivo* in the cells of *E. coli* K12 MG1655 and its deletion mutant PR202 Δ*dna*KJ14 containing hybrid plasmid with genes *lux*AB (pF2 or pXen4). Bacteria were grown at 28°C to the middle of the logarithmic phase. Then chloramphenicol (167 µg/ml) was added to the suspension of the cells to suppress the following synthesis of protein and the culture was transferred into a water bath at elevated temperature for inactivation of luciferase. Temperature and time of incubation for each luciferase were selected so that the final level of the enzymatic activity was 0.01-0.1% of the
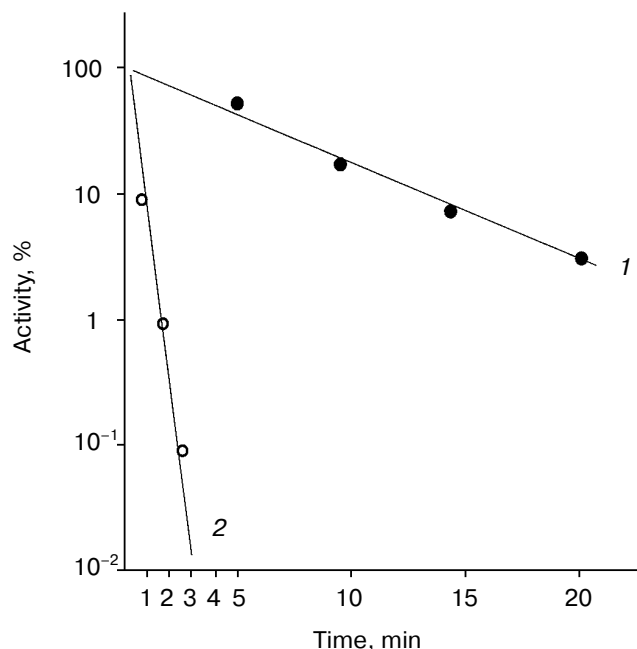


**Fig. 1.** Kinetics of thermal inactivation of *Ph. fischeri* and *Ph. luminescens* luciferases (*2* and *1*, respectively) *in vitro* at 43.5°C. The ordinate axis shows the enzymatic activity of luciferase in percent of the initial level. The abscissa axis is the time of thermal inactivation.

Rate constants for the enzymatic reaction and rate constants of thermal inactivation at 43.5°C for bacterial luciferases

| Luciferase | Rate constant for the enzymatic reaction $k_t$, sec$^{-1}$ | Rate constant of thermal inactivation $k_T$, min$^{-1}$ |
|---|---|---|
| *Ph. luminescens* | 0.009 | 0.067 |
| *Ph. fischeri* | 0.18 | 1.0 |

initial level. Refolding of inactivated luciferases was carried out at optimum temperatures: at room temperature or at 25°C for *Ph. fischeri* luciferase or at 35°C for *Ph. luminescens* luciferase. Figure 2 shows the time course of renaturation of luciferases (in percent of the initial level). As can be seen from the figure, refolding is completely lacking in the cells of the mutant strain PK202 Δ*dna*KJ. In the cells of *E. coli* MG1655 *dna*K$^+$ refolding takes place for both types of luciferases. However, in the case of thermolabile *Ph. fischeri* luciferase the degree of renaturation is markedly higher (80% of the initial level) than that

for thermostable *Ph. luminescens* luciferase (6-8% of the initial level). The kinetics of refolding of these luciferases are different: renaturation of thermolabile luciferase proceeds very quickly and reaches 5% level over 1-2 min, whereas refolding of thermostable luciferase is a substantially slower process (the degree of renaturation reaches the same level over 10-20 min).

**Influence of chaperones ClpA and ClpB on thermostability of luciferases.** The goal of the next series of the experiments was to study the kinetics of thermal inactivation of luciferases *in vivo* in *E. coli* *clp*A$^+$*clp*B$^+$ and *E. coli* containing mutations in genes *clp*A and *clp*B. Figure 3 shows the kinetic curves of thermal inactivation of *Ph. luminescens* luciferase (Fig. 3a) and *Ph. fischeri* luciferase (Fig. 3b) in the cells of *E. coli* 20250 *clp*A$^+$*clp*B$^+$, 22099 *clp*A$^-$*clp*B$^+$ and 22100 *clp*A$^+$*clp*B$^-$. We discovered two characteristic features of the kinetics of thermal inactivation: 1) the shape of the kinetic curves obtained in strains *clp*B$^+$ (*clp*A$^+$*clp*B$^+$ and *clp*A$^-$*clp*B$^+$) suggests the formation of a "thermoresistant fraction", the appearance of this fraction becoming especially notable on long incubation of the cells at high temperature (Fig. 3, a and b, curves *1* and *3*); 2) the thermostable luciferase from *Ph. luminescens* 22099 *clp*A$^-$*clp*B$^+$ reveals an enhanced thermosensitivity in the early stages of thermal inactivation (curve *3* in Fig. 3a); for thermolabile *Ph. fischeri*
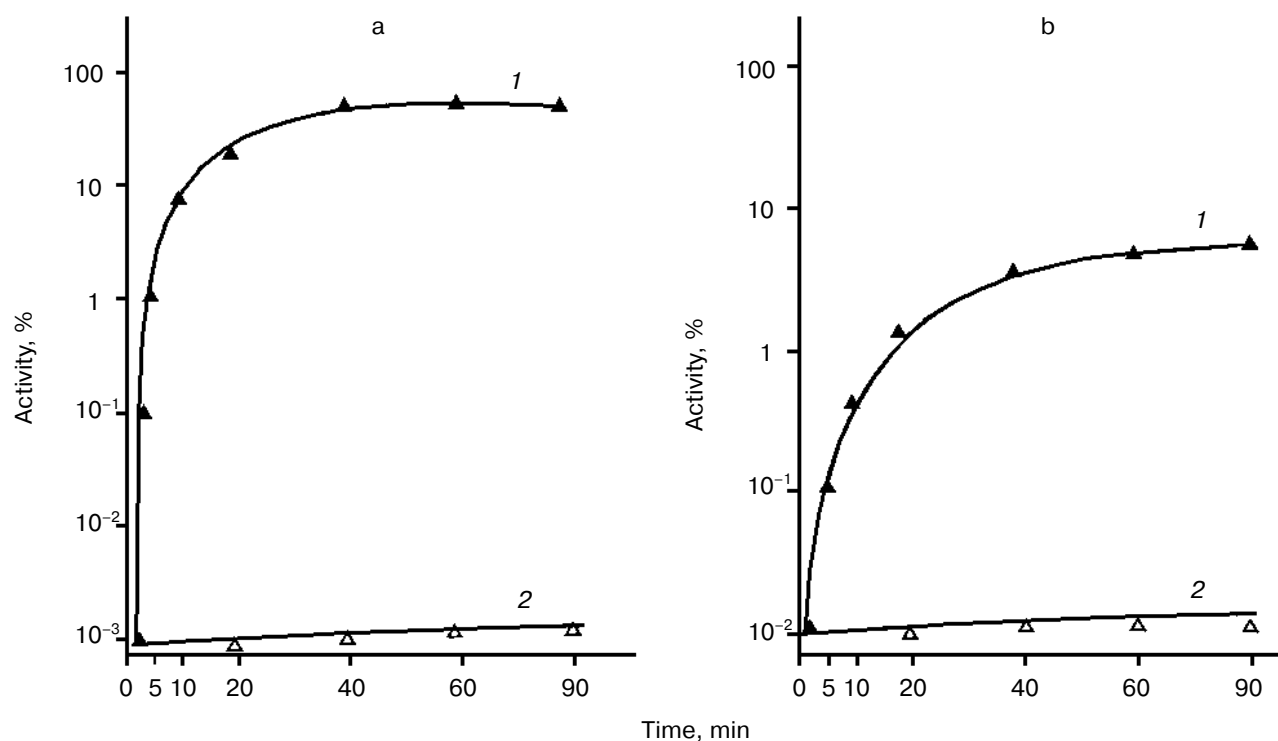


**Fig. 2.** Kinetics of refolding of thermoinactivated *Ph. fischeri* luciferase (a) and *Ph. luminescens* luciferase (b) in cells of *E. coli* MG1655 *dna*K$^+$ (*1*) and *E. coli* PK202 Δ*dna*KJ14 (*2*). The ordinate axis is the enzymatic activity of luciferase in percent of the initial level. The abscissa axis is the time of incubation at the temperature that is optimum for the particular luciferase (25°C for *Ph. fischeri* luciferase and 35°C for *Ph. luminescens* luciferase).

luciferase the effect of mutation in gene *clp*A on the shape of the inactivation curve is slight (curve *3* in Fig. 3b). In the case of *E. coli* mutant 22100 *clp*A$^+$*clp*B$^-$ the "resistant fraction" is lacking (curves *2* in Fig. 3, a and b). ClpB-dependent "thermoresistant fraction" is connected with DnaKJ-refolding, because in strain PK202 Δ*dna*KJ14 this fraction is also lacking (Fig. 4). Thus, one can conclude that the enhanced thermostability of *Ph. luminescens* luciferase observed on long incubation of the cells at high temperature is mainly determined by the combined action of chaperones DnaK−DnaJ−GrpE and ClpB and probably connected with refolding of aggregates of proteins [14-16]. It should be noted that in the case of thermolabile *Ph. fischeri* luciferase the portion of ClpB-dependent "resistant fraction" is a little higher. This is because refolding exerted by chaperones Hsp70 is more efficient (Fig. 2). As for the enhanced thermosensitivity of *Ph. luminescens* luciferase in the mutant strain 22099 *clp*A$^-$*clp*B$^+$, one can assume that chaperone ClpA has a protective effect during thermal inactivation. This effect coincides with the protective action of ClpA in the model system *in vitro* [29]. Mutation in gene *clp*X has no effect on the kinetics of thermal inactivation and refolding of luciferases (data not presented).

**The role of ATP in enhancing thermostability of luciferases *in vivo*.** As known, the effective action of chaperones ClpA and DnaKJE−ClpB requires large amounts of ATP [13-15, 30-33]. To reduce the content of ATP in the cells of *E. coli*, in the next series of the experiments we treated the cells with uncoupler of oxidative

phosphorylation CCCP. As can be seen from the data shown in Fig. 5, the addition of CCCP to the suspension of the cells 22099 *clp*A$^-$*clp*B$^+$ (pXen4) at the instant of the formation of the "resistant fraction" on the kinetic curve of inactivation of luciferase (45°C) results in the rapid disappearance of the latter: the residual activity of *Ph. luminescens* luciferase decreased by several orders of magnitude over 7-10 min (the addition of CCCP is designated by the arrow). Thus, refolding of luciferase exerted by DnaKJE−ClpB requires substantial amounts of ATP. CCCP acts in analogous fashion when this uncoupler is added to the cells of *E. coli clp*A$^-$*clp*B$^+$ and *clp*A$^+$*clp*B$^+$ in the early stages of inactivation of the enzyme. To eliminate ATP-dependent refolding exerted by chaperones DnaKJE, in the next series of experiments CCCP was added to the cells PK202 Δ*dna*KJ14 where refolding is lacking. Figure 4 shows the kinetic curves of thermal inactivation of *Ph. luminescens* luciferase in the strain of *E. coli* PK202 under standard conditions and in the case when CCCP was added in the early stages of thermal inactivation (43.5°C). Thermal inactivation shows a great increase in velocity and the kinetics of inactivation resemble those for the experiments *in vitro*. It should be noted that the addition of CCCP to the suspension of bacteria in L-broth at room temperature has no effect on the activity of luciferase and intensity of bioluminescence of the suspension throughout the experiment. The addition of CCCP results in the decrease in the concentration of ATP in the bacterial cells practically to the background level over some minutes at room
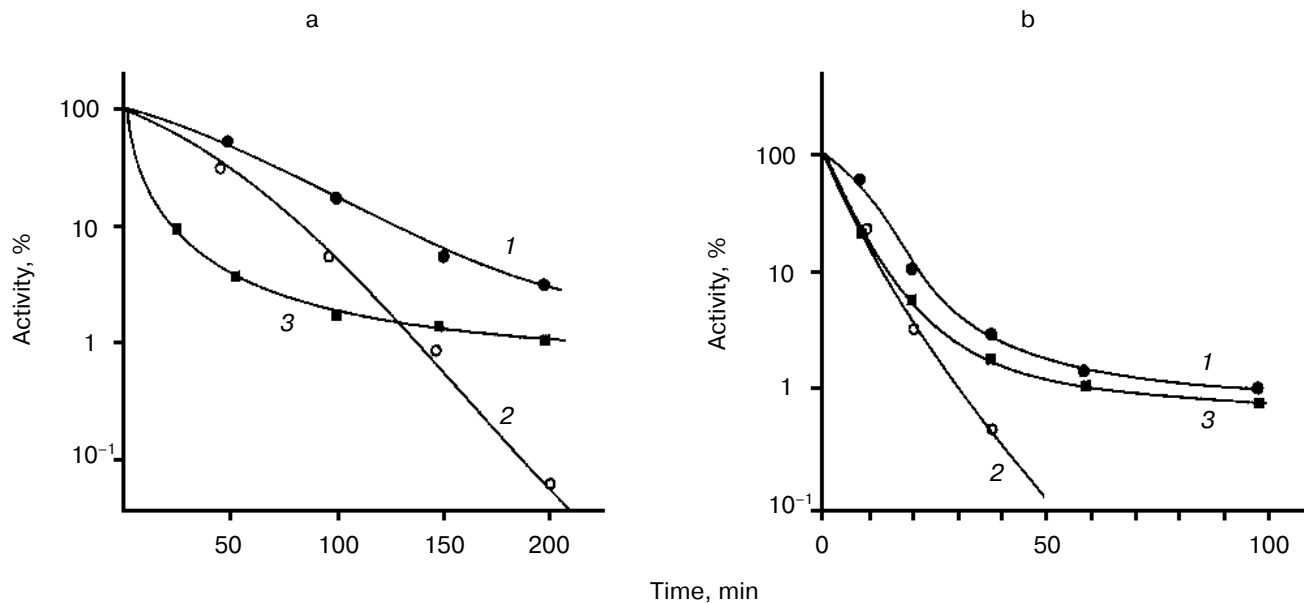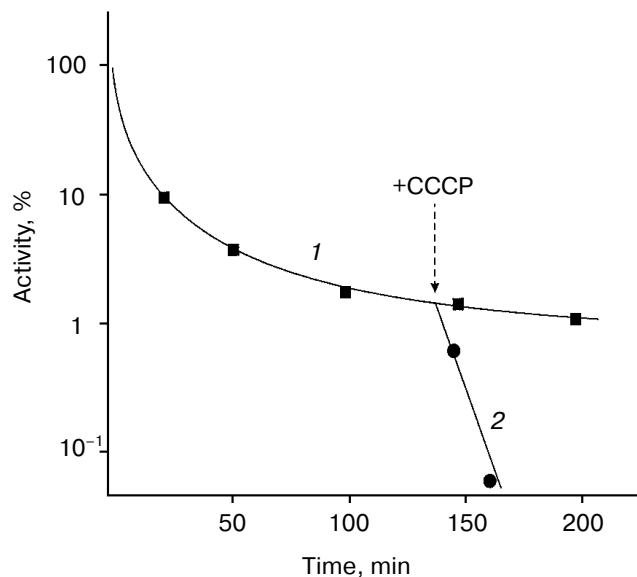


**Fig. 3.** Effect of mutations in genes *clp*A and *clp*B on the rate of thermal inactivation of *Ph. luminescens* (a) and *Ph. fischeri* (b) luciferases in the cells of *E. coli* 20250 *clp*A$^+$*clp*B$^+$ (*1*), *E. coli* 22100 *clp*A$^+$*clp*B$^-$ (*2*), and *E. coli* 22099 *clp*A$^-$*clp*B$^+$ (*3*). The cells were incubated in a water bath at 45.5 (a) or 38°C (b). The ordinate axis corresponds to the luciferase activity (intensity of bioluminescence) in percent of the initial level. Chloramphenicol (167 μg/ml) was added in the suspension of the cells to suppress synthesis of proteins.
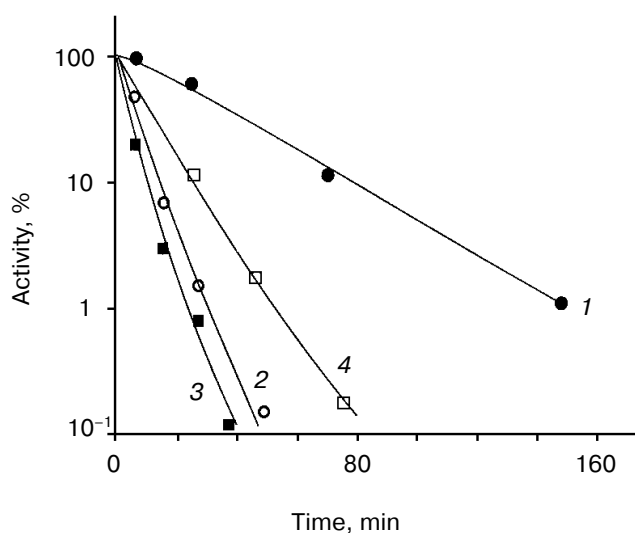
**Fig. 4.** Kinetics of thermal inactivation of *Ph. luminescens* luciferase in the cells of *E. coli* P202 Δ*dna*KJ at 43.5°C in the absence (*1*) and in the presence of 50 μM CCCP (*2*). Curve *3* corresponds to the isolated luciferase *in vitro*. Curve *4* was obtained in the presence of 50 μM CCCP and 0.6% glucose. The ordinate axis corresponds to the intensity of bioluminescence of the suspension of the cells in percent of the initial level.

**Fig. 5.** Effect of uncoupler of oxidative phosphorylation CCCP on the rate of thermal inactivation of *Ph. luminescens* luciferase in the cells of *E. coli* 22099 *clp*A⁻*clp*B⁺ in the region of the appearance of the "thermoresistant fraction" in the absence (*1*) and in the presence of 50 μM CCCP (*2*). The arrow indicates the point in time at which CCCP was added. Temperature of inactivation was 45.5°C. The ordinate axis corresponds to the intensity of bioluminescence of the suspension of the cells in percent of the initial level.

and elevated temperatures. Thus, one can conclude that the low level of ATP in the cell is crucial for chaperone-dependent refolding of the protein, but not for the bioluminescence reaction. Analogously, uncoupler of oxidative phosphorylation FCCP enhances the rate of thermal inactivation of *Ph. luminescens* luciferase in the cells PK202 (data not presented). The addition of glucose to L-broth decreases the efficiency of action of CCCP. This result may be ascribed to the additional synthesis of ATP in the glycolytic process, which is independent of ATP-synthases (curve *4* in Fig. 4).

As a working hypothesis, one can assume that there are two alternative pathways of renaturation of denatured *Ph. luminescens* and *Ph. fischeri* luciferases in the cells of *E. coli*. It is probable that thermolabile luciferase from *Ph. fischeri* has the higher affinity in respect to chaperone DnaK in comparison with ClpA. Therefore, the main pathway of renaturation of this protein should proceed with the participation of chaperones of the DnaK−DnaJ−GrpE system (direct refolding) or needs the additional participation of chaperone ClpB (disaggregation followed by refolding). It is likely that thermostable *Ph. luminescens* luciferase has enhanced affinity with respect to chaperone ClpA in comparison with DnaK. Therefore, for this enzyme DnaK-dependent refolding is of little importance. However the protein bound to chaperone ClpA does not aggregate. Subsequent hydrolysis of ATP provides release of denatured protein,

the latter most probably spontaneously refolding into the native structure.

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