Folding and refolding of thermolabile and thermostable bacterial luciferases: the role of DnaKJ heat-shock proteins

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Abstract Bacterial luciferases are highly suitable test substrates for the analysis of refolding of misfolded proteins, as they are structurally labile and loose activity at 42°C. Heat-denatured thermolabile *Vibrio fischeri* luciferase and thermostable *Photorhabdus luminescens* luciferase were used as substrates. We found that their reactivation requires the activity of the DnaK chaperone system. The DnaKJ chaperones were dispensable in vivo for de novo folding at 30°C of the luciferase, but essential for refolding after a heat-shock. The rate and yield of DnaKJ refolding of the *P. luminescens* thermostable luciferase were to a marked degree lower as compared with the *V. fischeri* thermolabile luciferase. The refolding activity of the DnaKJ chaperones was examined at various temperatures. Between 30 and 37°C, the refolding rates of the *V. fischeri* luciferase decreased and the reaction reached a complete arrest at temperatures above 40°C. The rate of DnaKJ-mediated refolding of the thermostable luciferase at first increased between 30 and 37°C and then decreased at the range of 37–44°C. We observed that the rate of DnaKJ-mediated refolding of the heat-denatured *P. luminescens* thermostable luciferase, but not of the thermolabile *V. fischeri* luciferase, decreased during the prolonged incubation at a high (47°C) temperature. The efficiency and reversibility of protein refolding arrest during and after heat-shock strongly depended on the stability of the DnaKJ-denatured luciferase complex. It is supposed that the thermostable luciferase is released during the heat-shock, whereas the thermolabile luciferase remained bound to the chaperone.

Key words: Chaperone; DnaK; DnaJ; Bacterial luciferase; Folding; Refolding

1. Introduction

Folding and assembly of cellular polypeptides is thought to require molecular chaperones as accessory factors [1–3]. The requirement has been demonstrated for several specific proteins [4–7] and is suggested by the association of chaperones with newly formed polypeptides [8,9]. Hsp70 molecular chaperones are ATPases which participate in many cellular pathways, including protein folding, translocation and proteolysis [9]. The heat-shock proteins DnaK, DnaJ and GrpE from *Escherichia coli* constitute a three component chaperone system that prevents the aggregation of denatured proteins and assists the refolding of proteins in an ATP-dependent manner [9,10]. They use the energy of ATP binding and hydrolysis to regulate their interactions with hydrophobic regions of unfolded proteins. In studies of the protein refolding in vitro by DnaK and its cohorts, DnaJ bound to the unfolded protein and prevented its aggregation but was unable to restore the native conformation [10,11]. For refolding to occur, interaction with DnaK was required, a process facilitated by DnaJ. The DnaK-unfolded protein complex must, in turn, dissociate to allow the completion of folding. GrpE acts at this dissociation step, facilitating the release of bound ADP and, consequently, the unfolded polypeptide from DnaK [12]. Firefly luciferase has been used as a model substrate for studying the chaperone requirements for protein folding and refolding [12–14]. The concerted action of DnaK, DnaJ and GrpE results in the refolding of denatured firefly luciferase in vivo and in vitro [15,16].

In the present work, we used bacterial luciferases. They were chosen for this study because of the extremely sensitive enzymatic assay, which permits monitoring the appearance of the native polypeptide in a cell. Unlike monomeric firefly luciferase, the luciferases from all bacterial species studied so far consist of two subunits, α and β, with molecular weights of about 40 000 and 35 000, respectively. The two subunits are highly homologous, but only the α subunit carries the active center. A role of the β subunit is not clear but it is essential for a high quantum yield reaction [17,18]. In the present work, the *lux*AB genes required for the expression of luminescence have been cloned from a terrestrial bacterium *Photorhabdus luminescens* and from a marine bacterium *Vibrio fischeri*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. coli K-12 MG1655 F^- (prototroph) and its temperature-sensitive chaperone mutant PK202 *dna*K14 *dna*J14 *dks::kan* were used in all experiments (these strains were kindly provided by Dr Elizabeth A. Craig, USA). The *dna*K deletion mutation strain PK202 carries a deletion of the entire *dna*K encoding sequence and 155 bp of the 5′-end of the *dna*J gene. Since part of the *dna*J gene was also deleted, the resulting construction is $DnaK^-DnaJ^-$ (this allele was named Δ*dna*KJ14 [19]). The strain *P. luminescens* ZM1 was kindly provided by Dr Alla P. Zarubina, M.V. Lomonosovs Moscow State University.

Bacteria were grown in L-broth and on the corresponding agarized medium at 30°C. The antibiotics chloramphenicol, kanamycin and ampicillin were used at a final concentration of 10, 40 and 100 μg/ml, respectively. The optical density (OD) of the bacterial suspension was measured at 590 nm using a KFK-2MP photocolorimeter.

2.2. DNA manipulation and plasmid construction

DNA digestion with restriction endonucleases, DNA isolation from an agarose gel and ligation were performed by standard procedures [20]. Plasmids were isolated using the wizard isolation kit (Promega, USA) as recommended by the manufacturers.

The *lux* genes required for the expression of luminescence have been cloned from a marine bacterium, *V. fischeri*, and from a terrestrial bacterium, *P. luminescens*. Hybrid plasmid pF2 was constructed using

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the pUC18 vector and contained *V. fischeri* MJ-1 strain *lux*A and *lux*B genes under the *lac* promoter [21].

A *P. luminescens* ZM1 library was generated by ligation of the *Eco*RI-restricted chromosomal DNA into the pUC18 plasmid. Approximately one out of 1000 colonies of transformed *E. coli* TG1 emitted light, indicating that the genes for luciferase (*lux*AB) and for the aldehyde synthesizing enzymes (*lux*CDE) were present. The pXen7 recombinant plasmid contains the *P. luminescens lux*CDABE genes in an *Eco*RI fragment of about 7 kb. Further restriction by *Cla*I and *Dra*I produced a 2.8 kb fragment that on ligation to the pBluescript II KS plasmid and expression in *E. coli* emitted a high level of luminescence on the addition of aldehyde, thus localizing *lux*A and *lux*B between the *Cla*I and *Dra*I sites at 2.6 and 5.4 kb on the restriction map. That recombinant plasmid was named pXen2.8.

2.3. Electrophoresis of proteins

Electrophoresis was performed by the Laemmli method [22]. Culture samples were harvested and whole-cell proteins (from preparations having equivalent ODs) were treated with 4×sample buffer (20% β-mercaptoethanol, 8% SDS, 0.25 M Tris-HCl, pH 8.6, 40% glycerol, 0.0001% bromophenol blue) and heated 5 min at 100°C. Ready samples were added to a 12% polyacrylamide gel (10 mg total protein per track) and run for 1.5 h at 250 V, 30 mA. Protein bands were detected by staining gels with Coomassie brilliant blue. The amount of chaperone proteins was calculated according to the data from scanning gels by an Ultrascan XL LKB.

2.4. Production and activity measurements of bacterial luciferases

In vivo measurements of the luciferase activity were done using a luminometer consisting of a FEU-85 photomultiplier and V2-15 microvoltmeter in special cells. 200 μl suspension was mixed with 4 μl of 0.001% *n*-decanal in ethanol for 30 s–1 min before measurement. The production of luciferase and heat-shock proteins was stopped by addition of chloramphenicol to a final concentration of 167 μg/ml. For heat inactivation of luciferase in vivo, the cultures were transferred to a 44–46°C shaking water bath for 5–30 min. For subsequent recovery, cultures were transferred back to lower temperatures (20–40°C).

3. Results and discussion

3.1. Thermal inactivation of luciferases

A comparison of the thermal stabilities of the *P. luminescens* and *V. fischeri* luciferases was carried out in *E. coli* PK202 Δ*dna*KJ14. *E. coli* PK202 cells carrying the plasmids pF2 or pXen2.8, containing *lux*AB genes, were grown in LB at 30°C to the mid- or late-logarithmic phase. Chloramphenicol (167 μg/ml) was added to block the de novo protein synthesis and the cell suspension was incubated in a water bath at 44°C. Culture aliquots of 200 μl each were transferred to the luminometer and the bioluminescence activity was measured as a function of the time and the values were plotted as a percentage of the initial activity. The *V. fischeri* luciferase is inactivated at 44°C with a τ_{10} of 1 min, whereas the *P. luminescens* luciferase is remarkably stable with a τ_{10} for the thermal inactivation of over 20 min.

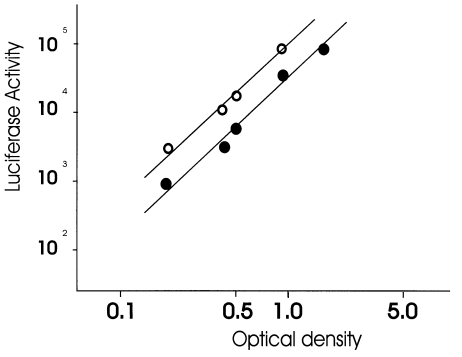

Fig. 1. De novo synthesis and folding of the *V. fischeri* luciferase. Cells of wild-type (MG1655) (●) and mutant (PK202) (○) strains, as indicated, containing plasmid pF2 were grown in LB medium at 30°C. The enzymatic activity was expressed as the intensity of bioluminescence in relative U. Values are from a representative experiment.

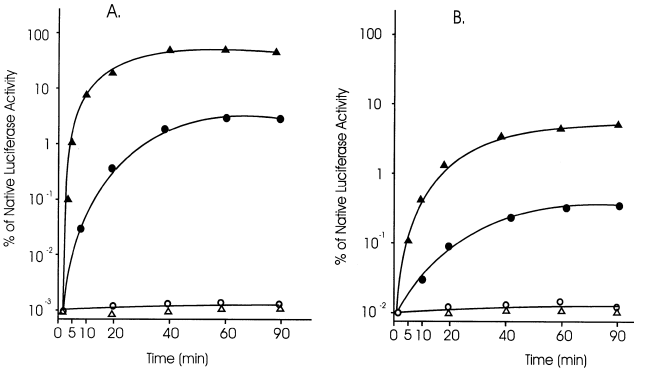

Fig. 2. The role of the DnaKJ chaperones in refolding of the heat-denatured bacterial luciferases. (A) *V. fischeri* luciferase, (B) *P. luminescens* luciferase. Cells of wild-type (MG1655) and PK202 Δ*dna*KJ14 strains containing the plasmids pF2 (A) and pXen2.8 (B) were grown in LB medium at 30°C to the mid-logarithmic phase. The luciferase production was followed by in vivo determination of the luciferase activity. Further protein synthesis was stopped by adding chloramphenicol to the final concentration of 167 μg/ml. For heat inactivation of the luciferase in vivo, the cultures were transferred to a 44°C shaking water bath for 5 min (A) and to 46°C for 15 min. (B) For subsequent recovery, cultures were transferred back to a 30°C (A) and 37°C (B) shaking water bath and assayed for luciferase reactivation. All activities were calculated as a percentage of the native luciferase activity. For the maximal DnaKJ level, MG1655 cells were at first incubated at 42°C for 30 min without chloramphenicol (preliminary heat-shock). A representative experiment of five is shown. (○) PK202, (△) PK202 (with preliminary heat-shock), (●) MG1655, (▲) MG1655 (with preliminary heat-shock).

3.2. DnaKJ are not essential for folding of luciferases at 30°C

The *V. fischeri* luciferase produced at 30°C in wild-type cells and the Δ*dna*KJ14 mutant accumulated to similar amounts and yielded similar activities (in Δ*dna*KJ14 even somewhat more) in time-resolved assays performed in vivo (Fig. 1). A similar result was observed for the *P. luminescens* luciferase (data not shown). Therefore, this finding rules out a strictly essential role for *E. coli* DnaK and DnaJ in the de novo folding of the bacterial luciferase at an intermediate temperature.

3.3. DnaKJ are essential for refolding of heat-inactivated bacterial luciferases

The ability of DnaK to prevent the aggregation of proteins in heat-shock conditions may be a prerequisite for their refolding under permissive conditions. This was tested using the thermolabile *V. fischeri* luciferase and the thermostable *P. luminescens* luciferase as test substrates. MG1655 and PK202 Δ*dna*KJ14 cells containing the pF2 or pXen2.8 plasmid were grown at 30°C to the mid-logarithmic phase. Further protein synthesis was blocked by adding chloramphenicol (167 μg/ml) and the cultures were exposed to a high temperature to inactive the proteins. Temperatures and incubation times were selected for every type of luciferase so that the level of inactivation reached 10^{-4}–10^{-5} (5–10 min at 44°C for the

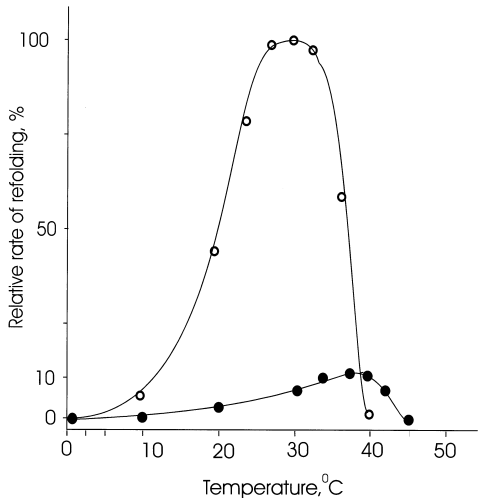

Fig. 3. The effect of temperature on the DnaKJ-mediated rate of bacterial luciferases refolding. *V. fischeri* luciferase (○), *P. luminescens* luciferase (●). Protein refolding at the indicated temperatures was initiated by the transfer of cell suspensions into water bathes with different temperateres and the time-dependent reactivation was measured. Maximum rates of DnaKJ-mediated refolding were calculated from the linear phase of the time curves of recovered enzymatic activities and plotted as a percentage of the rate of the *V. fischeri* luciferase refolding at 30°C. Each value is the mean of quadruplicate experiments.

V. fischeri luciferase and 15–20 min at 46°C for the *P. luminescens* luciferase). During the following recovery period at 20–37°C, heat-inactivated luciferases were reactivated in *dna*K$^+$ cells, while in Δ*dna*KJ14 cells no reactivation was observed (Fig. 2).

The DnaKJ levels control the efficiency of repair of heat-inactivated firefly luciferases [23]. We examined the efficiency of luciferase reactivation in strains with adjusted levels of DnaKJ. For this, before luciferase inactivating, cells were incubated for different time intervals at 42°C to increase the amount of DnaKJ (preliminary heat-shock). In non-stressed *E. coli* cells, there are estimated concentrations of 5 μM DnaK, 1 μM DnaJ and 1 μM GrpE [10,11]. The cellular concentration of DnaK increases 13-fold during a middle heat-shock [10,11,24]. In our experiments, the level of DnaKJ chaperones in cells increased from 2-fold (5 min of heat-shock at 42°C) to 10-fold (30 min of heat-shock at 42°C) as compared with the non-stressed cells. The refolding efficiency of the *V. fischeri* luciferase in the non-stressed cells was about 3–4% of the activity before the heat inactivation and was tightly correlated with the levels of DnaK and DnaJ, reaching 70–75% at 30 min of the preliminary heat-shock (Fig. 2A).

The rate and yield of refolding of the *P. luminescens* thermostable luciferase were strongly decreased as compared with the rate and yield of refolding of the *V. fischeri* luciferase: 0.3% for non-stressed cells and 8–9% for heat-shocked cells (Fig. 2B).

The refolding activity of the DnaKJ chaperones was examined at various temperatures. The DnaKJ-mediated refolding activity strongly depended on the temperature. The refolding rates of the *V. fischeri* luciferase decrease between 30 and 37°C and the reaction reaches a complete arrest above 40°C (Fig. 3). The rate of DnaKJ-mediated refolding of the *P. luminescens* thermostable luciferase increased between 30 and 37°C, then decreased between 37 and 42°C. The refolding temperature of 37°C was optimal for the thermostable luciferase (Fig. 3).

3.4. A prolonged incubation of cells at a high temperature (47°C) strongly decreases the rate and yield of refolding of the thermostable luciferase but not the thermolabile luciferase

During heat-shock, bacterial luciferases are the subject to unfolding and heat-shock chaperones are concomitantly synthesized in large amounts. Heat-induced exposure of hydrophobic residues from the core of thermolabile proteins may lead to protein aggregation, unless hydrophobic residues are allowed to interact with a chaperone surface. Here, we examined the protein refolding activity of DnaKJ chaperones in *E. coli* cells under a prolonged incubation at a high temperature (47°C). The protein synthesis was stopped by the addition of chloramphenicol. DnaKJ chaperones are thermostable proteins which remain unaffected by heat-shock conditions used in this study [25]. The rate and yield of DnaKJ-mediated refolding of heat-inactivated luciferases were found to gradually decrease as the incubation time at 47°C was increased. The protein refolding inhibition during incubation at 47°C was strongly expressed in the case of the thermostable luciferase (Fig. 4B) and weakly in the case of the thermolabile luci-

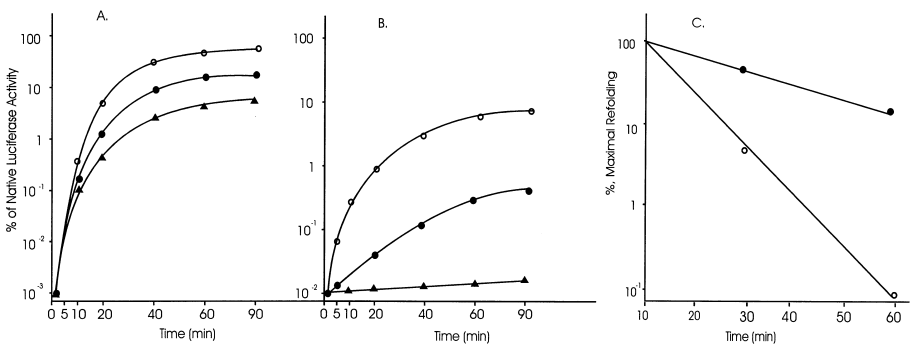

Fig. 4. Recovery of the enzymatic activity of bacterial luciferases after different periods of incubation at 47°C. (A) *V. fischeri* luciferase, (B) *P. luminescens* luciferase. Cells (after a preliminary heat-shock at 42°C, Fig. 2) were incubated with chloramphenicol (167 μg/ml) in a water bath at 47°C for 10 (○), 30 (●) and 60 (▲) min and then cooled down to 30°C for the *V. fischeri* luciferase and to 37°C for the *P. luminescens* luciferase. The time-dependent reactivation of the enzymatic activity (expressed as the intensity of bioluminescense) was measured. All activities were calculated as a percentage of the native luciferase activity. A representative experiment of four is shown. (C) The dependence of the maximum luciferase reactivation on incubation times at 47°C. (●) MG1655 (pF2), (○) MG1655 (pXen2.8). Maximum refolding values were taken from A and B. All values were calculated as a percentage of the initial maximal refolding (10 min at 47°C).

ferase (Fig. 4A). Hence, a denatured thermolabile protein can be arrested in a folding competent state during stress at 47°C and resumes DnaKJ-mediated refolding after the stress. A similar arrest of proteins in a folding competent state during heat-shock has been demonstrated in vitro in a case of the urea-denatured proteins [26].

This effect is clearly seen if one considers the maximum refolding values as a function of the incubation time at 47°C (Fig. 4C). We observe the inversion in the thermosensitivity of the proteins: the *V. fischeri* thermolabile luciferase appears a more thermostable enzyme as compared with the *P. luminescens* luciferase.

In conclusion, a lower level of refolding of the thermostable *P. luminescens* luciferase is determined presumably by a lack of ability of the unfolded protein to remain bound with DnaKJ chaperones during a heat-shock. As the result, it leads to the formation of irreversible aggregates. In strict contrast to the observed defect of refolding of the *P. luminescens* luciferase, the unfolded thermolabile *V. fischeri* luciferase remains bound with DnaKJ chaperones during heat-shock. This is essential for keeping the inactive and aggregation prone luciferase in a soluble state that is competent for refolding under permissive conditions.

We have obtained similar results for *Photobacterium leiognathi* thermolabile luciferase and *Vibrio harveyi* thermostable luciferase.

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References

[1] Bukau, B. and Horwich, A.L. (1998) Cell 92, 351–366.
[2] Gething, M.-J. and Sambrook, J. (1992) Nature 355, 33–45.
[3] Hartl, F.-U. (1996) Nature 381, 571–580.
[4] Buchberger, A., Schroder, H., Hesterkamp, T., Schonfeld, H., Sprang, S.R., Sambrook, J.F. and Gething, M.-J. (1993) Cell 75, 717–728.
[5] Buchberger, A., Schroder, H., Hesterkamp, T., Schonfeld, H.J. and Bukau, B. (1996) J. Mol. Biol. 261, 328–333.
[6] Pierpaoli, E.V., Sandmeier, E., Baici, A., Schonfeld, H.J., Gisler, S. and Christen, P. (1997) J. Mol. Biol. 269, 757–768.
[7] Zhu, X., Zhao, X., Burkholder, W., Gragerov, A., Ogata, C., Gottesman, M. and Hendrickson, W. (1996) Science 272, 1606–1614.
[8] Martin, J. and Hartl, F.-U. (1997) Curr. Opin. Struct. Biol. 7, 41–52.
[9] Rudiger, S., Buchberger, A. and Bukau, B. (1997) Nat. Struct. Biol. 4, 342–349.
[10] Szabo, A., Langer, T., Schroder, H., Flanagan, J., Bukau, B. and Hartl, F.-U. (1994) Proc. Natl. Acad. Sci. USA 91, 10345–10349.
[11] Bardwell, J.C.A., Tilly, K., Craig, E., Kings, J., Zylicz, M. and Georgopoulos, C. (1986) J. Biol. Chem. 261, 1782–1785.
[12] Schroder, H., Langer, T., Hartl, F.-U. and Bukau, B. (1993) EMBO J. 12, 4137–4144.
[13] Gamer, J., Multhaup, G., Tomayasu, T., McCarty, J.S., Rudiger, S., Schonfeld, H.J., Schirra, C., Bujard, H. and Bukau, B. (1996) EMBO J. 15, 607–617.
[14] Frydman, J., Nimmesgern, E., Ohtsuka, K. and Hartl, F.-U. (1994) Nature 370, 111–117.
[15] Hesterkamp, T. and Bukau, B. (1998) EMBO J. 17, 4818–4828.
[16] Goffin, L. and Georgopoulos, C. (1998) Mol. Microbiol. 30, 329–340.
[17] Fisher, A.J., Raushel, F.M., Baldwin, T.O. and Rayment, I. (1995) Biochemistry 34, 6581–6586.
[18] Baldwin, T.O., Christopher, J.A., Raushel, F.M., Sinclair, J.F., Ziegler, M.M., Fisher, A.J. and Rayment, I. (1995) Curr. Opin. Struct. Biol. 5, 798–809.
[19] Kang, P.J. and Craig, E.A. (1990) J. Bacteriol. 172, 2055–2064.
[20] Sambrook, J., Fritsch, F. and Maniatis, T.E. Molecular Cloning: a Laboratory Manual (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor. New York.
[21] Zavil'gel'skii, G.B. and Manukhov, I.V. (1994) Russ. J. Genet. 30, 297–300.
[22] Laemmli, U.K. (1970) Nature 227, 680–685.
[23] Tomoyasu, T., Ogura, T., Tatsuta, T. and Bukau, B. (1998) Mol. Microbiol. 30, 567–581.
[24] Goodsell, D.S. (1991) Trends Biochem. Sci. 16, 203–206.
[25] Reid, K.L. and Fin, A.L. (1996) Cell Stress Chaperones 1, 127–137.
[26] Diamant, S. and Goloubinoff, P. (1998) Biochemistry 37, 9688–9694.