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Research Article

The role of hsp70 in protection and repair of luciferase activity in vivo; experimental data and mathematical modelling

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Abstract. The stably transfected rat cell line HR24 expressing high levels of the inducible human hsp70 and its parental cell line Rat-1 were used for in vivo studies to analyse the role of hsp70 during thermal protein denaturation and the subsequent renaturation. In order to monitor denaturation and renaturation of a cellular protein in vivo, both cell lines were transiently transfected with firefly luciferase (Luc). The continuous monitoring of Luc activity during and after heat stress allowed a detailed analysis of the inactivation and reactivation kinetics in cells grown in monolayers. The aim of these studies was to distinguish a protective effect of increased hsp70 levels during heat shock-induced protein inactivation from a stimulation of reactivation. In this paper we show that in cells that are stably transfected with hsp70, thermal Luc inactivation decreased, and subsequent reactivation yielded higher activity levels, compared with the parental cells. The difference in early inactivation kinetics observed in the two cell lines suggests an immediate effect of the presence of an extra amount of hsp70 on enzyme inactivation. Using different mathematical models, the heat-induced inactivation and reactivation kinetics was compared with simulations of denaturation and renaturation. It is concluded that the model in which it is assumed that hsp70 is able to interact with partially denatured proteins, which did not yet lose their enzymatic activity, most optimally explains the experimental observations.

Key words. Heat shock; hsp70; mathematical model; luciferase; fibroblasts; luminescence.

Cells respond to supraoptimal temperatures with the synthesis of heat shock proteins (hsps), which are suggested to protect cells from damage by heat and assist in normalisation of cellular functions during recovery from stress (reviewed in [1–5]). A number of studies have indicated a role for hsp70 in cytoprotection. A decrease in the cell's ability to withstand a severe heat shock was observed when hsp70 was depleted by microinjection of antibodies specific to hsp70 [6] or when the expression of hsp70 was reduced by promoter competition [7]. In contrast, microinjection of hsp70 directly into Chinese hamster ovary (CHO) cells caused an increase in cellular resistance to heat shock [8]. In addition, transfection with inducible hsp70 in mammalian cells has been found to confer heat resistance [9, 10].

One of the concepts that emerged from the studies of hsp70 family members is their role in molecular chaperoning. As chaperones they have the capacity to bind to folding intermediates, misfolded or (partly) denatured proteins and prevent their irreversible denaturation. Furthermore, they assist in renaturation and correct

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refolding of misfolded proteins [2, 4, 11–13]. Although both a protective action on protein structure as well as a stimulation of repair of denatured proteins by hsp70 is suggested, it is unclear whether its main action is to prevent damage or to improve recovery [14, 15].

In this study we focussed on the role of hsp70 in heat-induced denaturation and renaturation of cytoplasmic proteins using firefly luciferase (Luc) as reporter of in vivo enzyme activity. In mammalian cells, Luc has been suggested to provide one of the best nontoxic and sensitive enzymes to study characteristics of the process of denaturation and renaturation of thermolabile proteins in the same temperature range as the onset of heat-induced cellular protein denaturation [16, 17]. Recently, the in vivo detection of Luc activity has been described in suspensions of plant cells [18]. By extending this method, we demonstrate that Luc is one of the enzymes which can be detected intracellularly, noninvasively and with high sensitivity, which allows the monitoring of detailed inactivation and reactivation kinetics in cultures of mammalian cells.

Usually, heat-induced inactivation and subsequent reactivation of Luc activity are supposed to represent denaturation and renaturation of cytoplasmic proteins. However, it has recently been suggested that at least some proteins can already be partly denatured without affecting their enzymatic activity [19]. This observation is of extreme importance for the role of hsps in cellular physiology under adverse conditions, since partly denatured but not yet inactivated enzymes may already be recognised by hsps. Binding of hsp70 to these partly denatured but still active enzymes may explain its protective action by preventing further denaturation and/or refolding to its native state.

Molecular protection might then be defined as preventing the inactivation of enzyme function, which does not necessarily imply preventing the partial denaturation of protein structure. In this context, molecular repair is defined as the reactivation of enzyme function, which implies refolding of heat-denatured protein structure.

Using Luc as a reporter enzyme, we asked whether hsp70 protects cells from heat-induced protein inactivation and/or whether hsp70 facilitates reactivation of Luc after heat shock treatment. To perform these investigations on the role of hsp70, stably transfected cells overexpressing human hsp70 (HR24 cells) and its parental cell line (Rat-1 cells) were used that have been described previously by Li et al. [10]. For the present studies the Rat-1 and HR24 cells have additionally been transfected with the pGL3 luciferase expression vector, which has a modified Luc gene to constitutively express the enzyme's activity in the cytoplasm. In these cells heat-induced inactivation and reactivation of Luc activity were monitored.

The data obtained were fitted to a mathematical model. In this model, the role of hsp70 in denaturation and renaturation of proteins was studied in relation to the inactivation and reactivation of Luc activity. Two options were analysed in which the relation between denaturation and inactivation was further differentiated. The analysis focussed on whether denaturation of a luciferase molecule immediately implies inactivation. In the first option it is supposed that denaturation does imply inactivation. In the second option it is supposed that denaturation may occur without immediate inactivation. The physiological importance is that complexation and protection by hsp70 of these partly denatured proteins takes place before enzymatic activity is affected.

One aspect in which both options were similar is that different degrees of denaturation are distinguished. Upon inactivation, the denaturation of a protein may proceed to different inactive intermediates that are characterised by increasing difficulty in reactivating, finally leading to unrecoverable aggregates. With respect to recovery of heat-inactivated luciferase activity, it has been suggested that highly denatured intermediates require more time and 'energy' for renaturation than the mild denatured ones [20, 21].

Earlier we proposed a model on the regulation of hsp70 synthesis which was used successfully to simulate the observations in experiments on hsp70 induction and the involvement of hsp70 in autoregulation of its synthesis [22]. In the present model we focussed on the role of hsp70 in protection and repair of Luc activity. The combination of experimental data and mathematical modelling suggest that Luc denaturation is a multistep process in which the thermostabilising effect of hsp70 is explained by a renaturation of denaturation intermediates, with some of the first-formed intermediates still showing enzymatic activity.

Materials and methods

Chemicals. Cell culture media were purchased from Gibco/Life Technologies (Alphen a/d Rijn, the Netherlands). Adenosine triphosphate (ATP) assay mix, Luc and ATP were obtained from Sigma (St. Louis, MO, USA) and luciferin from Applichem GmbH (Darmstadt, Germany).

Cell culture. Rat fibroblast cells (Rat-1) and the transfected Rat-1 cells (HR24), which constitutively express exogenous human hsp70, were gifts from Dr. G. C. Li [10]. The cells were routinely grown at 37 °C as monolayers in plastic flasks. Standard growth medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (Gibco/Life Technologies), 100 units/ml of potassium penicillin, 100 μg/ml of streptomycin sulphate and 10 mM HEPES.

Transfection. The Rat-1 and HR24 cells were transfected with the plasmid pGL3 Luc reporter vector (Promega) by the standard calcium phosphate method. The pGL3 Luc expression vector (Promega) has a modified Luc gene integrated under the control of the SV40 promoter with the SV40 late poly(A) signal and the SV enhancer sequence. Along with other changes (see technical manual pGL3 of Promega) the coding for the C-terminal tripeptide has been removed to eliminate peroxisome targeting of the expressed protein. The plasmid quantity used for transfection was 2 μg of pGL3 DNA per 8 cm^2 dish and 3 μg of carrier DNA (of pBluescript SK-, Stratagene). Transfection took place 24 h after plating, and cells were used for analysis 4–7 days after transfection.

Luc assay in intact cells. Luc activity of intact monolayers of pGL3 transfected cells was determined by placing a petri dish monolayer culture with 1 ml of culture medium which was supplemented with 0.1 mM luciferin in the temperature-controlled chamber of the photodetection system. The cell monolayer was approximately 7 cm below the photomultiplier tube window of the photon counter. The photon counter, in a single photon counting mode, was equipped with a Hamamatsu R550 photomultiplier tube (spectral response 280–850 nm, 1.5 kV) kept at -20 °C. Standard high performance photon-counting electronics consisting of a low-noise preamplifier, amplifier, discriminator and ratemeter were used. Under these conditions background or dark current of the photomultiplier amounted to 60–90 counts per second. Luciferin was added 15 min before the measurement. The in vivo photon emission of the cells could be followed for days at 37 °C.

Luc activity of lysed cells. Transfected monolayer cultures were rinsed twice with Hanks balanced salt solution (HBSS) and then lysed in a 1% Triton X-100 buffer containing 1 mM ATP, 0.05 mM luciferin, 20 mM tricine, pH 7.8, 5 mM DTT, 5 mM EDTA, 10 mM $MgSO_4$ and 0.5 mg/ml bovine serum albumin. Photon emission was measured immediately (± 2 s) after lysis of the cells at 25 °C. At this temperature the signal slowly declines ($\pm 0.2\%$ per second). The average values of the first 5 s of the measurements were used for quantification of Luc activity.

Heat shock. Transfected Rat-1 and HR24 cells were exposed to heat shocks by placing the culture dishes either in a waterbath in which the temperature was regulated within 0.1 °C or in the temperature-controlled chamber of the photodetection system which was regulated within 0.2 °C. Temperature equilibration of the cell cultures took about 1 min. The photodetection system allowed the quantitative measurements of luminescence either during the heat treatment itself at the chosen heat shock temperature or before and immediately after the heat treatment at 37 °C.

Cellular ATP levels. Rat-1 and HR24 cells were washed twice with phosphate buffered saline (PBS) and lysed in 250 μl of ATP detection mix (20 mM tricine, pH 7.8, 5 mM DTT, 5 mM EDTA, 10 mM $MgSO_4$, 1% Triton X-100, 0.5 mg/ml of bovine serum albumin and 0.5 μl of ATP assay mix containing both Luc and luciferin). Photon emission was measured at 25 °C. At this temperature a slow decline of the emission is discernible over a period of several minutes. Photon emission was normally measured for about 30 s, then 3 μl of 10 mM ATP was added as an internal standard.

Determination of cell survival. To establish the effect of heat treatments on cell survival, a clonal-assay method after a preplating procedure was applied as described before [23]. According to this procedure, monolayers of cells were trypsinised, and the cells were resuspended and inoculated in appropriate numbers. After attachment the cells were exposed to a heat shock. One week later, colony formation was determined and expressed as the relative survival, that is the plating efficiency of the treated cells divided by that of control (untreated) cells.

Separation of proteins and Western blotting. Cells were lysed and solubilized in sample buffer (5% SDS, 10% β-mercaptoethanol, 15% glycerol, 125 mM Tris-HCl, pH 6.8). For immunoblotting, equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (acrylamide 10%, bisacrylamide 0.27%), transferred to nitrocellulose and probed with anti-hsp68/70. Bound antibody was visualised by alkaline phosphatase-mediated colour development.

Results

Heat inactivation of Luc in Rat-1 and HR24 cells in vivo. A sensitive and continuous assay was developed to monitor Luc activity in intact Luc-transfected Rat-1 and HR24 cells. Eight hours after transfection of Rat-1 and HR24 cells, with the pGL3 Luc expression vector, Luc activity could be detected. The activity increased for several days and reached a plateau at 4–7 days after transfection (± 2000 photons/s detected). In this 'plateau phase' period cells were used for further studies. The thermostability of Luc during heat treatment was evaluated in both cell lines. The effect of different temperatures on Luc activity in Rat-1 and HR24 cells is shown in figure 1. The initial exponential decay of the Luc activity at the higher temperatures suggests a first-order reaction kinetics at the start of the inactivation process. Obviously, higher temperatures increase the rate of Luc inactivation in both cell lines. However, at a given temperature this rate is remarkably lower in the hsp70-overexpressing HR24 cells compared with the parental Rat-1 cells. In order to obtain the same inactivation rate in both cell types, HR24 cells must be

exposed to a heat shock temperature that is about 1 °C higher compared with Rat-1 cells. Figure 1b shows that the amount of inducible h-hsp70, which is synthesised constitutively in HR24 cells, is about the same as the amount of cognate hsp70, resulting in a total hsp70 level in HR24 cells which is twice as high as in the Rat-1 cells. In figure 2 (right panel), an Arrhenius plot of the initial inactivation rates of Luc in the two cell lines is presented. As these data fit a straight line, it seems to be justified to calculate the activation energy of this process which turned out to be 494 kJ in Rat-1 cells and 630 kJ in HR24 cells.

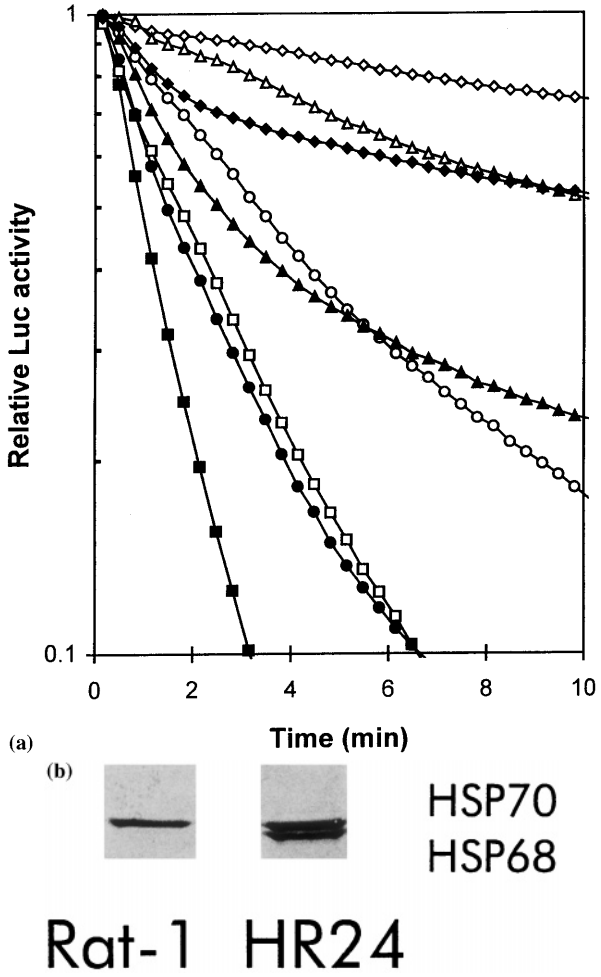

Figure 1. (*a*) Inactivation of Luc activity in transfected Rat-1 (closed symbols) and HR24 (open symbols) cells during heat treatments. Luc activity at 42 °C (◆, ◇), 43 °C (▲, △), 44 °C (●, ○), 45 °C (■, □) and (*b*) immunoblot of hsp68 and hsp70. For immunoblotting equal amounts of cellular protein of Rat-1 (first lane) and HR24 cells (second lane) were resolved by SDS-PAGE, transferred to nitroellulose and probed with anti-hsp68/70. Bound antibody was visualised by alkaline phosphatase-mediated colour development.

Thermal protein denaturation has often been associated with cell survival after heat stress. To test whether the observed protein inactivation of the exogeneous coded Luc is also a good reporter for cell survival after heat shock, clonal assays at a limited number of temperatures in both cell lines were determined. An Arrhenius plot of this survival is given in figure 2 (left panel). The results show that after the same heat shock temperature, HR24 cells are more resistant than Rat-1 cells. Again, a difference of about 1 °C is observed. The data indicate that there is a clear correlation between Luc inactivation at a given heat shock and survival of the cells exposed to this heat treatment. This correlation also indirectly supports the idea that Luc is a suitable reporter of thermal protein denaturation of the group of thermolabile proteins in the cell, which are critical for survival.

Since Luc is located inside the cells and its activity depends on the concentrations of both ATP and luciferin, we sought to verify whether the results obtained were influenced by changes in the intracellular concentrations of these compounds. The intracellular ATP concentrations of cells exposed to temperatures up to 45 °C were only slightly reduced (10%) in comparison with control cells. Furthermore, we measured Luc activity in cell lysates that were prepared immediately after a 10-min heat exposure as well as after a 1-h recovery period. Figure 3 shows that under these conditions, in which the ATP and luciferin concentrations are experimentally fixed, the Luc activity is directly proportional to the photon emission obtained from in vivo measurements at corresponding time points. We concluded that in our experiments in vivo Luc activity was not influenced by changes in the enzyme's substrate concentrations and that we thus actually measure in vivo Luc inactivation in the cell cultures.

Reactivation of heat-denatured Luc in Rat-1 and HR24 cells. In a second set of experiments, we investigated whether the in vivo system is suitable for monitoring the reactivation of heat-inactivated Luc. Cells were incubated for 10 min at various temperatures, and Luc activity was measured before and after thermal inactivation. Immediately following heat shock, the residual Luc activity was 1% to 25%, depending on the cell type and temperature tested. Reactivation of enzyme activity was monitored for 60 min at the cell's culturing temperature (37 °C). The two-phased recovery of Luc activity is shown in figure 4. The initial rapid recovery rate of Luc activity is gradually followed by a much slower recovery of enzyme activity. This recovery kinetic may be a result of the various degrees of loss of the native structure of Luc after heat shock. Luc with minor structural changes may reactivate in a rapid and easy way, whereas renaturation of Luc with an extended loss of tertiary structure and/or interaction with other denatured proteins may be a time-consuming process.

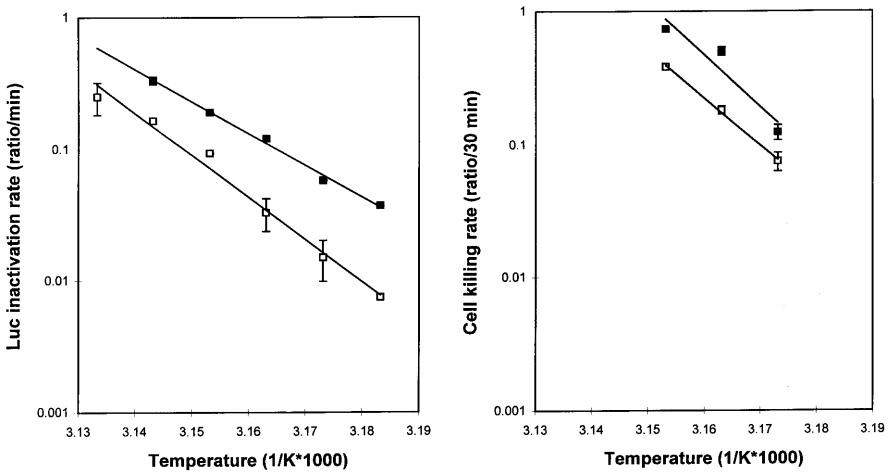

Figure 2. Arrhenius plots (left panel) of inactivation rate of Luc in transfected Rat-1 and HR24 cells at various temperatures, and (right panel) clonal survival of Rat-1 (closed symbols) and HR24 cells (open symbols) after a heat shock of 30 min at various temperatures.

After applying identical heat shocks to both Rat-1 and HR24 cells, Luc activity in the hsp70-overexpressing HR24 cells recovers to a higher extent than in the Rat-1 cells. This may be partly due to the fact that Luc is inactivated to a lesser degree in HR24 cells. Indeed, the recovery of Luc activity in both cell types is almost identical when comparing the results obtained with heat shocks which invoke an equal loss of Luc activity (e.g. when reactivation after a heat shock at 43 °C in Rat-1 is compared with that after a heat shock at 44 °C in HR24 cells), as shown in figure 4.

The recovery of Luc activity does not only depend on the result of reactivation of existing Luc. De novo synthesis of Luc may also be of importance. To determine whether the reactivation kinetics is influenced by de novo Luc synthesis, two approaches can be followed either determining the rate of de novo synthesis or by inhibiting new synthesis during reactivation.

To estimate the rate of Luc synthesis, we determined the half-life of the Luc protein, since the steady-state level of Luc depends both on its synthesis rate and its half-life. At the time of performing the experiments, Luc activity in the cells had reached a steady state. The half-life of Luc was estimated by determining the decrease in Luc activity in the presence of 5 μM cycloheximide (an inhibitor of protein synthesis) during 7 h. After 7 h about 80% of the initial activity was still observed in vivo and in vitro. From these data it could be estimated that in these cells, the half-life of Luc is 28 h and that its rate of synthesis can be estimated to be only 2.5% per hour of the amount of Luc present under normal culturing conditions. Since protein synthesis also decreases after heat shock, de novo synthesis of Luc following heat shock can thus only have a very minor contribution to the increase of Luc activity during the first hour of the recovery period.

We did not study the effect of protein synthesis inhibition during protein renaturation. Inhibition of protein

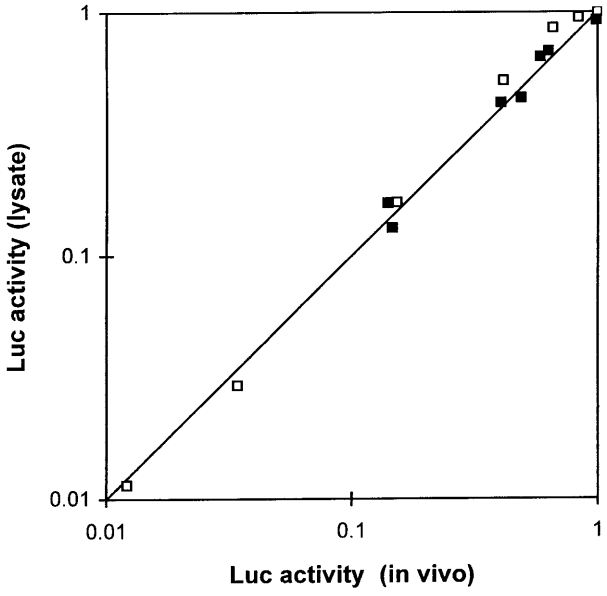

Figure 3. Luc activity in cell lysates compared with in vivo Luc activity. Cells were heat-shocked (10 min, at various temperatures), and Luc activity was measured immediately (□) or after a 1-h recovery period (■). The activity was expressed as the fraction of the activity at 37 °C (for in vivo and in vitro ± 2000 and ± 5000 counts per second, respectively).

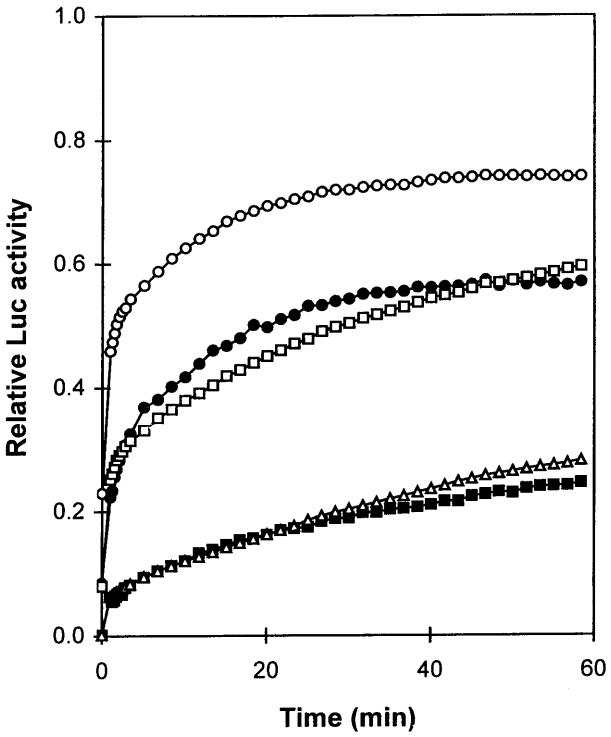

Figure 4. Renaturation kinetics of Luc activity in Rat-1 (closed symbols) and HR24 (open symbols) cells after a 10-min heat shock at 43 °C (●, ○), 44 °C (■, □) and 45 °C (△).

synthesis may interfere with renaturation processes since one of the physiological functions of the hsp70 family is the chaperoning of nascent and newly born polypeptides. Inhibition of protein synthesis could thus well lead to changes in the free amount of hsp70 and consequently in renaturation characteristics of heat-denatured proteins.

Modelling the role of hsp70 in heat inactivation and subsequent recovery of the Luc activity. To better understand the role of hsp70s in protecting heat inactivation of proteins and/or the subsequent recovery of this activity, two mathematical models were developed. In both models denaturation and renaturation processes of Luc are described in which inactivation of Luc forms part of the denaturation process. In the first model this inactivation of Luc is proposed to occur with the very first denaturation step, whereas in the second model minor conformational changes of Luc may occur without inactivation of the enzyme. This still active conformation of the enzyme is proposed to have an increased affinity to bind to hsp70. This is in agreement with suggestions that partial denaturation does nor necessarily imply inactivation of Luc [19, 24]. The biological processes, assumed to take place in the model, are a limited number of denaturation and renaturation cycles (fig. 5A,B for the first and second model, respectively).

A number of assumptions concerning the 'biological' processes involved were made in order to keep the models as simple as possible. Denaturation of a protein is usually described as a multistep process [4, 19]. Renaturation may therefore occur from several denaturation intermediates of the same protein. From our experimental data on renaturation of Luc, a fast and a slower recovery can be discerned (fig. 4) which could represent a renaturation from at least two different denaturation intermediates. Furthermore, our data indicate that recovery of the enzyme activity never reaches the 100% value at the time scale studied. This could mean that not all denatured Luc can be renaturated. Part of the irreversible denatured Luc molecules will probably be targeted for degradation. This means that a denatured Luc form can exist which cannot be renaturated.

The biological models were used as the basis of the present mathematical models. Since the denaturation/renaturation cycle in both mathematical models is repeated (three or four times) using the same types of formulas, we only present the formulas for one cycle. In the first steps of the denaturation process, the native protein (Luc1) changes its conformation (Luc2), resulting in an increased binding capacity of hsp70. In the first model this change in structure equals inactivation of the enzyme; in the second model this is not the case. The conversion of Luc1 to Luc2 is assumed to follow first-order reaction kinetics. The rate of this reaction is obviously temperature-dependent. The activation energy of the inactivation of Luc in the Rat-1 cells as calculated was used to define the temperature dependence of the reaction rates. Thus, choosing the reaction rate at a certain temperature determines the rates at the other temperatures:

$$\frac{d[luc_1]}{dt} = -k_1 x[luc_1] \tag{1}$$

$$k_1 = A_1\, e^{-E_{a1}/RT} \tag{2}$$

The binding of hsp to Luc2 is assumed to be a simple equilibrium reaction in which the reaction rates are relatively fast compared with those of the denaturation and renaturation process. This reaction can thus be characterised by its K value. Furthermore, it is assumed that the K values are the same for the different forms of Luc (except for the native Luc1 form, which we assumed to have no affinity for hsp):

$$K_m = \frac{[luc2 * hsp]}{[luc2][hsp]} \tag{3}$$

The renaturation process, which is equivalent to the conversion of the Luc2 * hsp complex to Luc1 and hsp,

is considered to be a first-order reaction. In our models this process is assumed not to be dependent on the temperature in the range used, and the reaction can be characterised by its conversion rate. We realise that this representation may be too simple, but we omitted the temperature dependency for this reaction to keep the model as straightforward as possible.

$$\frac{d[luc2 * hsp]}{dt} = -k2x[luc2 * hsp] \quad (4)$$

For the model, some assumptions about the biological parameters were made. With regard to the interactions that take place between hsp70 and hsc70, on the one hand, and native and denatured proteins, on the other, we assumed that there is no functional difference between hsp70 and hsc70, and their amounts in the cell may be added. From autoradiography data using SDS-PAGE to separate steady-state labelled proteins, the amount of hsp70 in the Rat-1 cells was estimated to be 0.7% of the cellular protein. The amount of hsp70 in the HR24 cells was estimated to be 1.4% of the cellular protein. This amount is about twice as high as in the Rat-1 cells and includes the cognate protein as well as the plasmid-coded hsp.

Further, we assumed that, in the time period studied, the total level of hsp70 does not significantly change, that the amount of Luc in the cell does not notably change due to protein synthesis or protein turnover, that no spontaneous renaturation of denatured Luc occurs and that Luc can be used as a model protein for the class of thermolabile proteins denaturing in the range up to 45 °C. Cell survival is known to be profoundly affected after prolonged exposure at this temperature. Using differential scanning calorimetry data, Lepock et al. [25] showed that about 5% of the denaturation of cellular proteins occurs below 45 °C. We assumed therefore that Luc represents 5% of the cellular proteins.

Based on the previous assumptions analyses were aimed at finding parameter sets for both models in which the calculated data best fitted the experimental data. The most optimal fit between experimental data and calculated data with either one of the models was defined as the minimisation of the least-squares values. One parameter set for each of the two models was chosen in such a way that they constituted a best fit for the kinetics and temperature dependence of the denaturation as well as the kinetics of the renaturation process. The differential equations describing the production of reactants were solved numerically. For each time step Δt, the concentrations of Luc forms and their complexes with hsp were determined. This was repeated

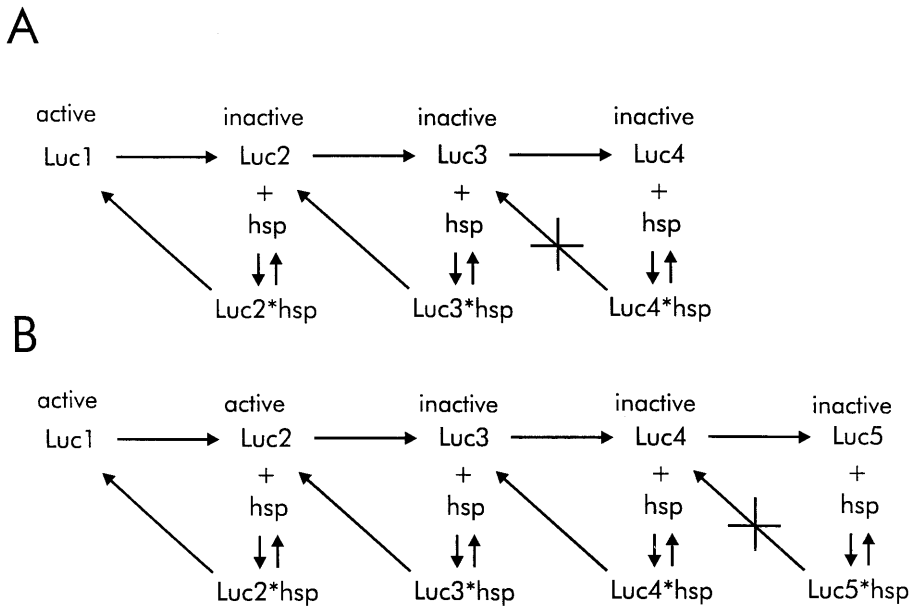

Figure 5. Model of the Luc denaturation and renaturation. (*A*) Luc1, native protein; Luc2, protein with a loss of structure resulting in an absence of enzyme activity; Luc3, protein with an increased loss of structure but which can still be renaturated; Luc4, denatured protein with heavy loss of structure which cannot be reactivated. (*B*) Luc1, native protein; Luc2, protein with a loss of structure but not resulting in loss of enzyme activity; Luc3, protein with a loss of structure resulting in an absence of enzyme activity; Luc4, protein with an increased loss of structure but which can still be renaturated; Luc5, denatured protein with heavy loss of structure which cannot be reactivated.

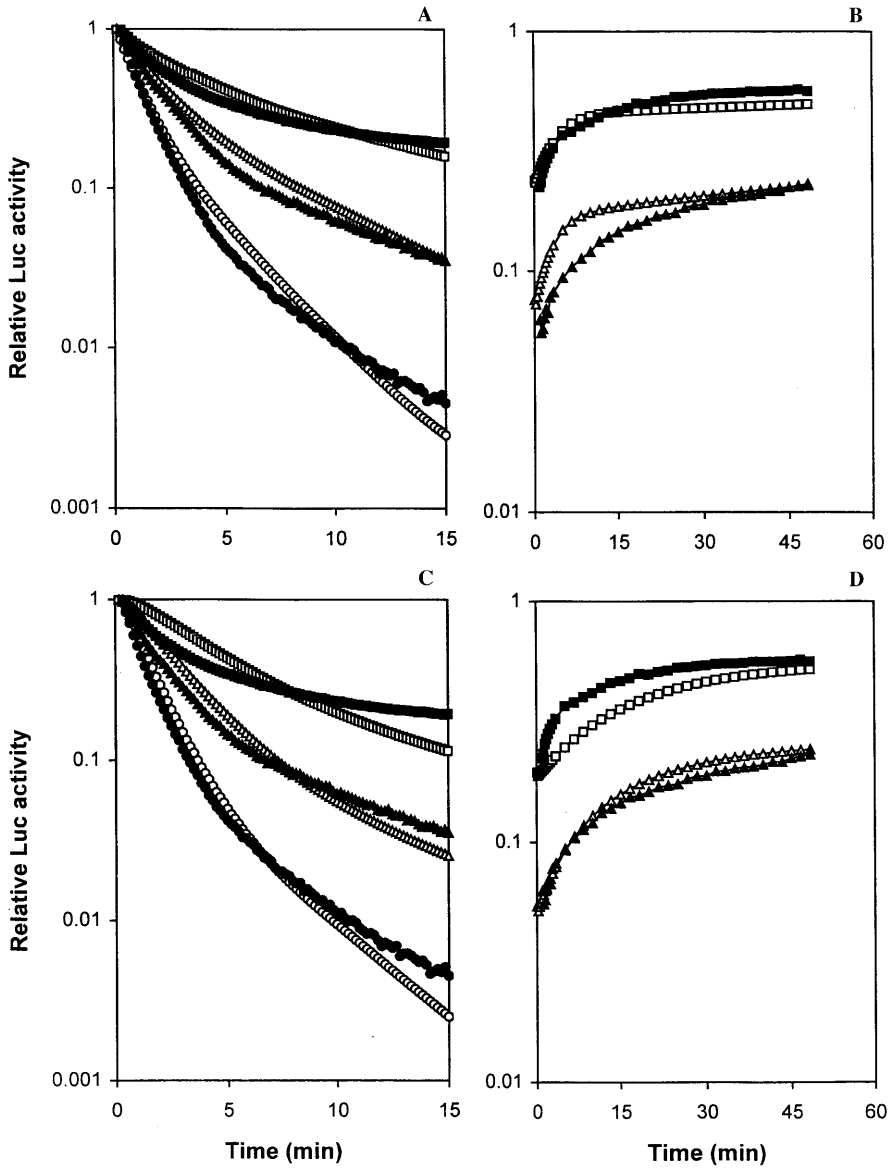

Figure 6. Denaturation (A + C) and renaturation (B + D) of luciferase in Rat-1 cells at various temperatures. The Luc activity of these cells was measured (closed symbols) and calculated (open symbols), using the first model and the model parameters in table 1 (A + B) or the second model and the model parameters in table 2 (C + D), during incubation for 15 min at 43 °C (■, □), 44 °C (▲, △) and at 45 °C (●, ○), and during a 1-h period at the normal culturing temperature after a 10-min heat treatment at 43 °C (■, □) and 44 °C (▲, △).

until the final time tend was reached. All calculations were checked to verify that the value chosen for the time interval Δt was small enough in order to make the results independent of Δt.

A comparison between the experimentally measured Luc activity and the calculated activities in the Rat-1 cells for the first model is shown for both inactivation and reactivation in figure 6A and B, respectively. In calculating these data, the parameter set of table 1 was used. The comparison between the experimental data in Rat-1 cells and the activities calculated according to the second model are shown in figure 6C and D, respectively. For these data the parameter set of table 2 was used.

As can be observed in this figure, parameter sets could be identified for both models that predict to a high degree both the kinetics and temperature dependence of the denaturation process as well as the kinetics of renat-

Table 1. Model parameter values of the three conversion cycles of Luc.

	Luc1–Luc2 cycle	Luc2–Luc3 cycle	Luc3–Luc4 cycle
k1 (min^{-1})	0.277289	0.216022	$<10^{-6}$
k2 (min^{-1})	1.103903	0.015205	0

K_m values for all the cycles were chosen to be 20 μM. The k1 values are given for 43 °C. The k1 values for the other temperatures were calculated using the Arrhenius equation with the parameters derived from figure 2 (left panel).

uration. Moreover, we can conclude, from the data sets of tables 1 and 2 that the formation of a Luc form that cannot be reactivated is not an essential part of the model, since the best fit of the calculated data is obtained with minute formation rates of these forms.

Using the same parameter sets for the HR24 cells, in which the hsp70 (r-hsc70 + h-hsp70) content was estimated to be 1.4% of the cellular protein, we calculated the denaturation and renaturation of Luc in these cells. In figure 7A and B the experimental and calculated values for the in- and reactivation of Luc in the HR24 cells are given for the first model, and in figure 7C and D for the second model. Here also the predicted values for both models fit the experimental data set to a high degree.

When the inactivation and reactivation characteristics between Rat-1 and HR24 cells (figs 6 and 7) are compared, it can be observed that a higher concentration of hsp70 in HR24 cells results in decreased inactivation of Luc during heat shock and a higher reactivation in the subsequent period.

Until now, both models describe the overall denaturation and renaturation process equally well. However, when considering the early phase of the denaturation process of the experimental data in more detail, we must conclude that Rat-1 and HR24 cells almost instantly differ in their Luc inactivation rates after temperature elevation. This suggests an immediate effect of hsp70 on the inactivation of Luc (fig. 8A). To study this in more detail, we compared the denaturation of Luc in the Rat-1 and HR24 cell lines for the two models in this early phase. The data of the first model showed that the Rat-1 and HR24 cells start to differ in their denaturation rate only after a substantial amount of inactivated Luc has been formed (fig. 8B). The data of the second model, in which interaction of hsp with the still active Luc1 form was assumed, showed an almost immediate effect of the presence of extra hsp70 (fig. 8C), although at lower temperatures to a lesser extent than found in the experimental data.

These results suggest that the second model, in which hsps are able to interact with Luc in an initial state of denaturation—a form in which Luc is proposed to maintain its activity—is a better representation of processes taking place during and after heat shock than the model in which hsps are only able to bind to inactivated forms of Luc. Thus it is proposed that real protection of enzyme activity during heat shock may occur when hsps can interact with proteins that are partly denatured but still maintain their enzymatic activity.

Discussion

In this paper we compared the heat-induced inactivation of Luc activity and its subsequent reactivation in the stably transfected cell line HR24 expressing high levels of hsp70 with the kinetics of these processes in its parental cell line, Rat-1 cells [10]. Specifically, we focussed on the question whether overexpression of hsp70 protects cells from heat-induced protein inactivation and/or facilitates reactivation of Luc during and after heat shock.

The combination of experimental data and mathematical modelling suggests that Luc denaturation is a multistep process in which the thermostabilising effect of hsp70 is due to renaturation of denaturation intermediates, with some of the earliest-formed intermediates still showing enzymatic activity.

In this sense, on the molecular level, protection can be (re)defined as the result of the interaction of hsp70 with partly denatured but not yet inactivated proteins, preventing further unfolding/denaturation or even initiating renaturation. Repair could then indicate the renaturation of inactivated proteins, a process that already occurs during heat treatment. This is in contrast to the use of the term 'protection' in the sense of less

Table 2. Model parameter values of the four conversion cycles of Luc.

	Luc1–Luc2 cycle	Luc2–Luc3 cycle	Luc3–Luc4 cycle	Luc4–Luc5 cycle
k1 (min^{-1})	1.211013	0.315583	0.140106	$<10^{-6}$
k2 (min^{-1})	2.316115	0.273376	0.002669	0

K_m values for all the cycles were chosen to be 20 μM. The k1 values are given for 43 °C. The k1 values for the other temperatures were calculated using the Arrhenius equation with the parameters derived from figure 2 (left panel).

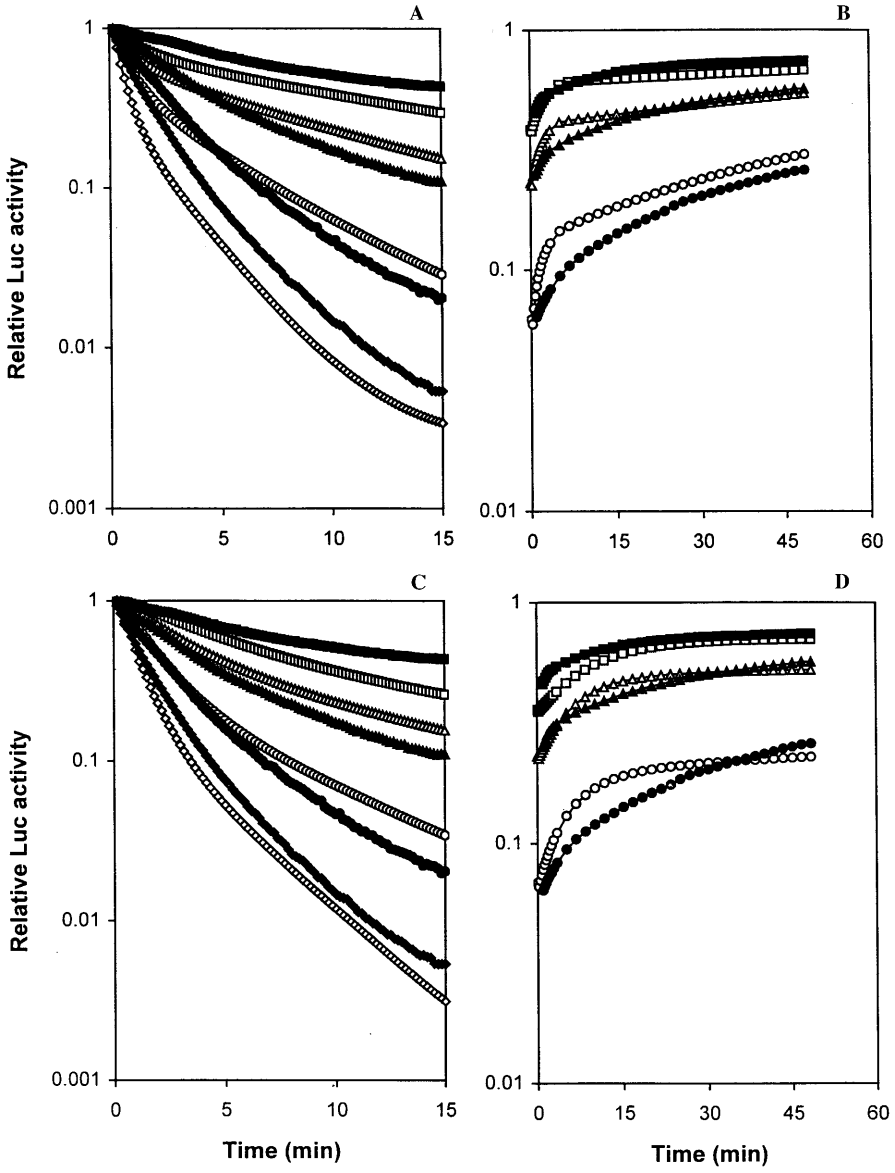

Figure 7. Denaturation (A + C) and renaturation (B + D) of Luc in HR24 cells at various temperatures. The Luc activity of these cells was measured (closed symbols) and calculated (open symbols), using the first model and the model parameters in table 1 (A + B) or the second model and the model parameters in table 2 (C + D), during incubation for 15 min at 43 °C (■, □), 44 °C (▲, △), 45 °C (●, ○) and at 46 °C (◆, ◇), and during a 1-h period at the normal culturing temperature after a 10-min heat treatment at 43 °C (■, □), 44 °C (▲, △) and 45 °C (●, ○).

damage obtained after heat shock, irrespective of the molecular processes taking place. In our model the protective action of hsp70 could be explained by its ability to bind to denatured proteins and its role in their renaturation.

It has been suggested that hsp70 transiently associates with short linear peptide segments of folding intermediates, usually short hydrophobic regions of six to nine amino acids [26, 27]. Such an association may prevent aggregation due to shielding of exposed 'sticky' hydrophobic sites and assists in refolding. Therefore, even in cells that overexpress hsp70, it can be assumed that not all cellular proteins have an interaction with hsp70, in order to prevent denaturation. However, in the earliest stage of denaturation, when enzyme activity may not yet be affected, an interaction with hsp70 can occur.

In this respect, Wood [24] suggested that the activity of Luc might be maintained under mildly denaturing con-

ditions that partly unfold its polypeptide structure. Jaenicke [19, 28] recently described the behaviour of partly denatured domainlike proteins. In the simplest case of two-domain proteins, an intermediate in unfolding was suggested containing one domain still intact and the other unfolded. When the active site is present in the intact domain, changes in the structure of the other domain are not necessarily reflected in a change in enzyme activity [19] but might cause association with hsp70, thus preventing further denaturation and assisting in refolding.

The molecular structure of Luc was shown to contain two densely packed domains connected by a single peptide backbone [29]. Both domains contribute to the active site with a highly conserved sequence of several amino acids located at opposite sides of the cleft between the two domains. This cleft is far too big to accommodate the substrates and to allow simultaneous interaction of the conserved surfaces. Therefore, it was suggested that the two domains come together during the course of the reaction to sandwich the substrates. This mechanism might progressively be affected upon increasing denaturation, thereby losing its enzyme activity.

With respect to the mathematical model, denaturation is suggested to occur in a number of steps. This is in agreement with the model forwarded by Herbst et al. [20]. They showed that the changes in enzymatic activity of Luc could be described as compatible with a four-state model according to N-In1-In2-U, where N is a native enzymatically active Luc, In1 and In2 are partially unfolded inactive intermediates and U is the completely denatured and unfolded polypeptide. In1 represent more native intermediates with a reduced tendency to aggregate and which renatures more easily, whereas In2 represents the nonnative intermediates that have a high tendency to aggregate and renature less easily.

Since denaturation of a protein is usually described as a multistep process [19, 30], renaturation may therefore occur from several denaturation intermediates of the same protein. Zocchi [31] recently showed that stepwise folding and unfolding of proteins follows deterministic pathways. Herbst et al. [20] showed that the time course of Luc reactivation in vitro does not follow first-order kinetics. They observed that at least two exponentials are required to describe the reactivation kinetics, a faster first phase in the minute time range and a slower second phase that takes hours. Our experimental data of Luc reactivation in vivo are in agreement with their observations; both a fast and a slower recovery can be discerned (fig. 4), which could represent renaturation from at least two different denaturation intermediates. Interestingly, Herbst et al. [21] showed that the more unfolded intermediate aggregated much more rapidly than less unfolded intermediates. Similar observations were described by Ranson et al. [32] with respect to the denaturation of mitochondrial malate dehydrogenase. They discerned two states, the first of which is slowly reversible and the second irreversible.

The question related to the role of hsp70 in improving recovery has been frequently discussed. Upon recovery from stress, both the solubility and the activity of many aggregated proteins can partially be recovered [2, 17,

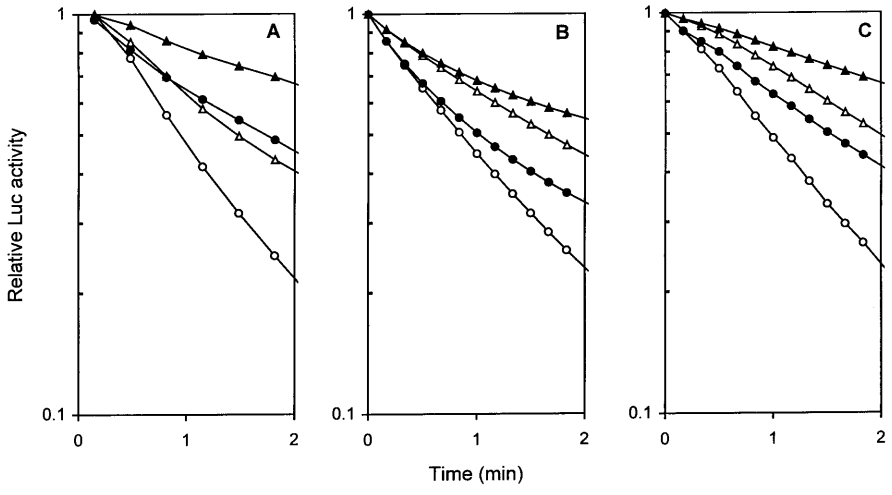

Figure 8. The initial denaturation of Luc in Rat-1 (open symbols) and HR24 cells (closed symbols). The Luc activity of these cells was (*A*) measured or calculated using either (*B*) the first model and the model parameters in table 1, or (*C*) the second model and the model parameters in table 2, at 44 °C (▲, △) and 45 °C (●, ○).

33]. After heat stress, the activity of the substrate can be restored upon addition of ATP and/or supplementation of certain components of the chaperone system [34–37]. In the present study we showed that when Luc is inactivated to the same degree in HR24 and Rat-1 cells, the same reactivation kinetics are observed, suggesting that hsp70 is not involved or rate-limiting in this process. This is in agreement with the observations of Stege et al. [38], who detected no difference in the disaggregation of nuclear proteins between HR24 and Rat-1 cells after heat shock that caused a similar degree of aggregation in these cell lines.

However, according to our model, this does not necessarily imply that hsp70 plays no role of any importance in reactivation and renaturation of proteins. Luc could be damaged or denatured to a higher degree in HR24 compared with Rat-1 cells, even when the degree of Luc inactivation is the same. This is the result of the 1 °C higher heat shock temperature to which the HR24 cells must be exposed. Since more extensive denatured intermediates (in HR24) will be renatured at a higher expense than less denatured intermediates (in Rat-1), this implies that the same reactivation kinetics could reflect an enhanced degree of renaturation in the cells overexpressing hsp70. In order to acquire full protein remodelling activity of the various heat shock-induced denatured and/or aggregated intermediates, it has frequently been suggested that hsp70 requires cooperation with other hsps, cochaperones, factors or binding proteins [39–42]. Also, for renaturation and reactivation of Luc, an increasing number of additional factors are described, such as hsp40, chaperonin (hsp60), hsp90, Hip, Hop, Bag-1, Ydj-1 and so on [18, 33, 36, 37, 41, 43, 44].

The cooperation of a number of chaparones (DnaK, DnaJ and GrpE) in an ATP hydrolysis-dependent reaction cycle to reactivate Luc in *Escherichia coli* was described by Schröder et al. [36] and Szabe et al. [37]. For fully efficient refolding they reported that several rounds of ATP-dependent interactions of denatured Luc with different hsps are required. Although additional factors are required to obtain fully efficient repair facilities, the level of hsp70 in Rat-1 cells still appears to be a limiting factor for protection and reactivation, since cell survival in the hsp70-overexpressing HR24 cells is increased [10].

The aim of our studies was to further analyse the role of hsp70 during stress and during recovery of adverse conditions. We believe that the present model system in which the kinetics of heat-induced inactivation and reactivation of luciferase is monitored in vivo is well suited for this purpose. Furthermore, the possibility of comparing experimental data with simulations of these processes obtained by mathematical modelling proved useful in further understanding the action of hsps in protection and repair at the molecular level.

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