The Hsp70 chaperone system maintains high concentrations of active proteins and suppresses ATP consumption during heat shock

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Abstract Hsp70 chaperones assist protein folding by cycling between the ATP-bound T state with low affinity for substrates and the ADP-bound R state with high affinity for substrates. The transition from the T to R state is catalyzed by the synergistic action of the substrate and DnaJ cochaperones. The reverse transition from the R state to the T state is accelerated by the nucleotide exchange factor GrpE. These two processes, T-to-R and R-to-T conversion, are affected differently by temperature change. Here we modeled Hsp70-mediated protein folding under permanent and transient heat shock based on published experimental data. Our simulation results were in agreement with in vitro wild-type *Escherichia coli* chaperone experimental data at 25°C and reflected R-to-T ratio dynamics in response to temperature effects. Our simulation results suggested that the chaperone system evolved naturally to maintain the concentration of active protein as high as possible during heat shock, even at the cost of recovered activity after return to optimal growth conditions. They also revealed that the chaperone system evolved to suppress ATP consumption at non-optimal high growing temperatures.

Keywords GrpE · Heat-shock · Hsp70 · Protein folding · Systems biology · Temperature

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Introduction

Proteins have to maintain a correct three-dimensional structure to be functional. Failure to fold correctly can lead to aggregation, which is associated with diseases such as Alzheimer's disease, Huntington's disease, and type II diabetes (Dobson 1999; Radford 2000; Dobson 2006). Although all the information for the precise three-dimensional structure of a protein is encoded in its amino acid sequence (Anfinsen 1973), in vivo many proteins depend on the assistance of molecular chaperones such as Hsp70 and Hsp60 for folding from the nascent or denatured state into their correct structures (Gething and Sambrook 1992; Langer et al. 1992; Bukau et al. 2000). The most efficient chaperone system for preventing aggregation in vivo is the Hsp70 system (Mogk et al. 1999). At least 50% of all proteins are estimated to bind to Hsp70 during biosynthesis in *Escherichia coli* (Agashe and Hartl 2000; Feldman and Frydman 2000). The kinetics of the Hsp70 chaperone system have been studied most extensively for the *E. coli* Hsp70 homologue DnaK with its J-domain cochaperone DnaJ and the nucleotide exchange factor GrpE (Langer et al. 1992; Mayer et al. 2000a; Mayer et al. 2001; Hartl and Hayer-Hartl 2002).

These studies found that central to DnaK's chaperoning action is the nucleotide-regulated transition between two conformational states, the ATP-bound T state with high association and dissociation rates but low affinity for substrates, and the ADP-bound R state with two and three orders of magnitude lower association and dissociation rates but high affinity for substrates (Fig. 1A) (Schmid et al. 1994; Gisler et al. 1998; Mayer et al. 2000b). Spontaneous transition between the two states is extremely slow but is stimulated

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Temperature up-shift will change the rate constants for the individual partial reactions of the Hsp70 refolding system according to the law of Arrhenius, except in the case of the interaction of DnaK with its nucleotide exchange factor GrpE. GrpE catalyzes the conversion of DnaK from the ADP-bound R state, with a high affinity for substrates but low substrate association and dissociation rates, to the ATP-bound T state, with a low substrate affinity but high association and dissociation rates. Experimental data show that this GrpE-catalyzed conversion is temperature sensitive and does not follow the Arrhenius temperature

dependence (Grimshaw et al. 2001; Grimshaw et al. 2003). Upon temperature up-shift, GrpE unfolds and becomes inactive. When the temperature returns to the physiological range, GrpE folds back spontaneously and becomes active again. A similar temperature-dependent unfolding of GrpE is also observed in the Hsp70 system of *Thermus thermophilus*, albeit at much higher temperatures (Groemping and Reinstein 2001), demonstrating that this property of the nucleotide exchange factor might be an important regulatory element of the chaperone system. Grimshaw et al. proposed that the reversible unfolding of GrpE, which leads to a reduction in the nucleotide exchange rate and therefore supposedly to a reduction in the substrate dissociation rate at elevated temperatures, serves to prevent substrate release under conditions when the substrate cannot fold back into the native state (Grimshaw et al. 2001; Grimshaw et al. 2003). Such a mechanism supposedly prevents futile ATPase cycles of the chaperone system. Although these considerations seem logical, they do not take into account the fact that the rate constants of all other reactions of the refolding cycle also change with temperature. How the chaperone system functions at elevated temperature, and for which property it was optimized in evolution, is therefore still enigmatic.

Organisms cope with heat shock by increasing the expression of heat shock genes encoding chaperones and proteases (Gross 1999). In *E. coli* this heat shock response is mediated by the heat shock transcription factor σ^{32} , the activity and stability of which increase dramatically during the heat shock response, leading to an approximately 10-fold increase in heat shock gene transcription 5 to 10 min after temperature up-shift. The heat shock response is followed by a shut-off phase whereby heat shock gene transcription is reduced to a new steady state level. The result of this transient increase in heat shock gene expression is a 2- to 3-fold increase in the levels of DnaK and its cochaperones. To quantitatively analyze the consequences of this increase in chaperone levels on the proportions of active, denatured, and aggregated proteins, and to determine the importance of the time-point of increase in chaperone levels, we implemented the heat shock response in our model.

Because of the limitations of biochemical and biophysical techniques, it is necessary to combine computer simulation with these methods to study heat shock systems. In this work, we analyzed the effect of temperature on the whole chaperone system using a top-down kinetic simulation strategy. The refolding result of simulation by our model at 25°C was validated by comparison with in vitro refolding data. We

discussed how other simulation results at 25°C and 45°C might be validated experimentally. Our model showed that increasing temperature increased the R to T ratio, in agreement with experimental findings. We compared Hsp70 chaperone-aided refolding at 25°C and 45°C and found that the intrinsic kinetic property of the chaperone system tends to slow down loss of active protein and suppress ATP consumption during heat shock at the cost of refolding yield after return to physiological temperatures.

Materials and methods

Reaction and parameters

All reactions and parameters used in this computer model were either based on the published literature or taken from our published Hsp70 model (Hu et al. 2006). Reaction equations, including parameters and references, are given in Table 1, together with the buffer conditions used in the measurements. Parameters determined in different laboratories using buffers different from the one used for firefly luciferase were checked exemplarily in the appropriate buffer and found to be similar. For reactions that started with the same reactants but had alternative products depending on a probability factor, rate constants were assigned to accommodate the desired probability. The reaction volume was set to the typical volume of *E. coli* cytoplasm.

Model development

To construct a kinetic model for the chaperone-assisted refolding of a denatured protein, the refolding process was dissected into its elemental chemical equations (Fig. 1B and Table 1), with sets of kinetic parameters for the individual steps. The elemental chemical equations and the parameters used were based on the published literature and were taken from our published Hsp70 model (Hu et al. 2006). In our model substrate protein has three free states: denatured state (S^D), aggregated state (S^A), and native state (S^N). To allow for scaling of the refolding kinetics, we assigned a probability value expressing the likelihood with which a denatured protein molecule would be refolded in a single cycle of binding and release by the chaperones. Thereby, ATP hydrolysis was considered crucial and only a substrate that was bound to DnaK in the ATP state and released subsequent to ATP hydrolysis had a chance of refolding to the native state. Because of data

Table 1 Reactions in the kinetic model of the DnaK chaperone system

No. ^a	Reaction	Rate	References
1	$2 \text{ DnaJ} \rightarrow \text{DnaJ}_2$	$10^5 \text{ M}^{-1} \text{ s}^{-1}$	Estimated ^b
2	$\text{DnaJ}_2 \rightarrow 2 \text{ DnaJ}$	10^{-4} s^{-1}	Estimated ^b
3	$\text{DnaK} + \text{ATP} \rightarrow \text{DnaK} \cdot \text{ATP}$	$1.3 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$	Russell et al. (1998) A
4	$\text{DnaK} \cdot \text{ATP} \rightarrow \text{DnaK} + \text{ATP}$	$1.33 \cdot 10^{-4} \text{ s}^{-1}$	Russell et al (1998) A
5	$\text{DnaK} + \text{ADP} \rightarrow \text{DnaK} \cdot \text{ADP}$	$2.67 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$	Russell et al. (1998) A
6	$\text{DnaK} \cdot \text{ADP} \rightarrow \text{DnaK} + \text{ADP}$	0.022 s^{-1}	Theyssen et al. (1996), Russell et al. (1998) A, B
7	$\text{DnaK} \cdot \text{ATP} \rightarrow \text{DnaK} \cdot \text{ADP} + \text{Pi}$	$6 \cdot 10^{-4} \text{ s}^{-1}$	McCarty et al. (1995) C
8	$\text{GrpE}_2 \cdot \text{DnaK} \rightarrow \text{DnaK} + \text{GrpE}_2$	0.033 s^{-1}	Chesnokova et al. (2003) C
9	$\text{GrpE}_2 \cdot \text{DnaK} \cdot \text{ATP} \rightarrow \text{GrpE}_2 \cdot \text{DnaK} + \text{ATP}$	127 s^{-1}	Estimated based on Packschies et al. (1997) B
10	$\text{GrpE}_2 \cdot \text{DnaK} \cdot \text{ATP} \rightarrow \text{DnaK} \cdot \text{ATP} + \text{GrpE}_2$	0.1 s^{-1}	Estimated based on Fig. 4a in Chesnokova et al. (2003)
S1	$\text{DnaJ}_2 + \text{S}^{\text{D}} \rightarrow \text{S}^{\text{D}} \cdot \text{DnaJ}_2$	$3.3 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$	Gamer et al. (1996) D
S2	$\text{S}^{\text{D}} \cdot \text{DnaJ}_2 \rightarrow \text{DnaJ}_2 + \text{S}^{\text{D}}$	0.0062 s^{-1}	Gamer et al. (1996) D
S3	$\text{DnaK} \cdot \text{ATP} \cdot \text{S}^{\text{D}} \rightarrow \text{DnaK} \cdot \text{ADP} \cdot \text{S}^{\text{D}} + \text{Pi}$	0.001 s^{-1}	Laufen et al. (1999, Mayer et al. (2000b) C
S4	$\text{DnaK} \cdot \text{ADP} \cdot \text{S}^{\text{D}} \rightarrow \text{DnaK} \cdot \text{S}^{\text{D}} + \text{ADP}$	0.022 s^{-1}	Estimated to be similar to #6
S5	$\text{DnaK} \cdot \text{S}^{\text{D}} + \text{ATP} \rightarrow \text{DnaK} \cdot \text{ATP} \cdot \text{S}^{\text{D}}$	$1.3 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$	Estimated to be similar to #3
S6	$\text{DnaK} \cdot \text{ATP} \cdot \text{S}^{\text{D}} + \text{DnaJ}_2 \rightarrow \text{DnaK} \cdot \text{ATP} \cdot \text{S}^{\text{D}} \cdot \text{DnaJ}_2$	$3.3 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$	Estimated to be similar to #S1
S7	$\text{DnaK} \cdot \text{ATP} \cdot \text{S}^{\text{D}} \cdot \text{DnaJ}_2 \rightarrow \text{DnaK} \cdot \text{ATP} \cdot \text{S}^{\text{D}} + \text{DnaJ}_2$	0.0062 s^{-1}	Estimated to be similar to #S2
S8	$\text{DnaK} \cdot \text{ADP} \cdot \text{S}^{\text{D}} \cdot \text{DnaJ}_2 \rightarrow \text{DnaK} \cdot \text{ADP} \cdot \text{S}^{\text{D}} + \text{DnaJ}_2$	0.0062 s^{-1}	Estimated to be similar to #S2
S9	$\text{S}^{\text{D}} \rightarrow \text{S}^{\text{A}}$	$7 \cdot 10^{-4} \text{ s}^{-1}$	Estimated
T to R	$\text{DnaK} \cdot \text{ATP} \cdot \text{S}^{\text{D}} \cdot \text{DnaJ}_2 \rightarrow \text{DnaK} \cdot \text{ADP} \cdot \text{S}^{\text{D}} \cdot \text{DnaJ}_2 + \text{Pi}$	1.8 s^{-1}	Laufen et al. (1999) C
R to T1	$\text{GrpE}_2 + \text{DnaK} \cdot \text{ADP} + \text{ATP} \rightarrow \text{GrpE}_2 \cdot \text{DnaK} \cdot \text{ATP} + \text{ADP}$	$1.1 \cdot 10^9 \text{ M}^{-2} \text{ s}^{-1}$	Grimshaw et al. (2001)
R to T2	$\text{GrpE}_2 + \text{DnaK} \cdot \text{ADP} \cdot \text{DnaJ}_2 \cdot \text{S}^{\text{D}} + \text{ATP} \rightarrow \text{GrpE}_2 \cdot \text{DnaK} \cdot \text{ATP} \cdot \text{S}^{\text{D}} + \text{DnaJ}_2 + \text{ADP}$	$1.1 \cdot 10^9 \text{ M}^{-2} \text{ s}^{-1}$	Grimshaw et al. (2001)
R to T3	$\text{GrpE}_2 + \text{DnaK} \cdot \text{ADP} \cdot \text{S}^{\text{D}} + \text{ATP} \rightarrow \text{GrpE}_2 \cdot \text{DnaK} \cdot \text{ATP} \cdot \text{S}^{\text{D}} + \text{ADP}$	$1.1 \cdot 10^9 \text{ M}^{-2} \text{ s}^{-1}$	Grimshaw et al. (2001)
K _{on} ATP1	$\text{DnaK} \cdot \text{ATP} + \text{S}^{\text{D}} \cdot \text{DnaJ}_2 \rightarrow \text{DnaK} \cdot \text{ATP} \cdot \text{S}^{\text{D}} \cdot \text{DnaJ}_2$	$4.5 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$	Estimated to be similar to #K _{on} ATP2
K _{on} ATP2	$\text{DnaK} \cdot \text{ATP} + \text{S}^{\text{D}} \rightarrow \text{DnaK} \cdot \text{ATP} \cdot \text{S}^{\text{D}}$	$4.5 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$	Schmid et al. (1994), Gisler et al. (1998) E
K _{off} ATP1	$\text{DnaK} \cdot \text{ATP} \cdot \text{S}^{\text{D}} \cdot \text{DnaJ}_2 \rightarrow \text{DnaK} \cdot \text{ATP} + \text{S}^{\text{D}} \cdot \text{DnaJ}_2$	2 s^{-1}	Estimated to be similar to #K _{off} ATP2
K _{off} ATP2	$\text{DnaK} \cdot \text{ATP} \cdot \text{S}^{\text{D}} \rightarrow \text{DnaK} \cdot \text{ATP} + \text{S}^{\text{D}}$	2 s^{-1}	Schmid et al. (1994), Gisler et al. (1998) E
K _{on} ADP1	$\text{DnaK} \cdot \text{ADP} + \text{S}^{\text{D}} \rightarrow \text{DnaK} \cdot \text{ADP} \cdot \text{S}^{\text{D}}$	$1000 \text{ M}^{-1} \text{ s}^{-1}$	Estimated based on Mayer et al. (2000b) ^c C

Table 1 continued

No. ^a	Reaction	Rate	References
Fold	$S^D \rightarrow S^N$	$2.3 \cdot 10^{-4} \text{ s}^{-1}$	Calculated from Herbst et al. (1997)
K_{offADP1}	$\text{DnaK} \cdot \text{ADP} \cdot S^D \rightarrow \text{DnaK} \cdot \text{ADP} + S^D$	$4.7 \cdot 10^{-4} \text{ s}^{-1}$	Mayer et al. (2000b) C
Fold 1	$\text{GrpE}_2 \cdot \text{DnaK} \cdot \text{ATP} \cdot S^D \rightarrow S^D + \text{GrpE}_2 + \text{DnaK} \cdot \text{ATP}$	Depend on probability	
Fold 2	$\text{DnaK} \cdot \text{ADP} \cdot S^D \rightarrow \text{DnaK} \cdot \text{ADP} + S^D$	Depend on probability	

^a All reactions designated by S contain substrates. Reactions designated by RtoT, K_{onATP} , K_{offATP} , K_{onADP} , and K_{offADP} are reactions involving R to T state conversion, and binding and release of substrate to DnaKATP or ADP, respectively. Fold 1/2, reactions to introduce the refolding probability per chaperone cycle

^b The association and dissociation rates for DnaJ dimerization were estimated to fit the concentration dependence of the luciferase refolding reaction at low DnaJ concentrations

^c The association rate of a denatured protein to DnaK·ADP was estimated to be in between the association rate of a peptide and the rate for a native protein. Buffer conditions: A, 40 mM HEPES/KOH, pH 7.6, 11 mM Mg-acetate, 200 mM K-glutamate; B, 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 100 mM KCl, 2 mM EDTA, 2 mM dithioerythritol; C, 25 mM HEPES-KOH (pH 7.6), 5 mM MgCl₂, 50 mM KCl; D, 25 mM HEPES/KOH pH 7.0, 5 mM MgCl₂, 50 mM KCl, 5 mM 2-mercaptoethanol, and 0.005% Tween 20; E, 25 mM HEPES/NaOH pH 7.0, 5 mM MgCl₂, 100 mM KCl

limitations, we assumed that the presence of a third molecule in one complex would not affect the binding and dissociation of the two other molecules in the same complex (see Table 1). The model was validated by simulating the refolding of 1000 molecules of denatured proteins at 25°C and then comparing the results to experimental data on the refolding of denatured firefly luciferase (Fig. 2).

Effect of temperature on reaction parameters

To model the temperature shift, we first plotted the corresponding parameters versus temperature (Farr and Witt 1999; Grimshaw et al. 2001) and fitted a polynomial function to the data points (Fig. 3). All the rate constants could be fitted nicely within the temperature ranges reported. Next, we classified the

reactions in our model into different groups according to the data available in Fig. 3. There were 3 reactions for R to T conversion, 2 reactions for substrate binding to DnaK·ATP (i.e. interaction with S^D and $\text{DnaJ} \cdot S^D$), 2 reactions for substrate release from DnaK·ATP complexes, 1 reaction for substrate binding to DnaK·ADP and 1 for the reverse reaction (Table 1). For each reaction in the same group, we assumed that the same rate applied. There were still 20 reactions for which the temperature dependencies were not known. We assumed that these 20 reactions followed the standard reaction rate—temperature law for chemical reactions, which gives a doubling or tripling in rate every 10°C. A temperature shift from 25 to 45°C therefore leads to an approximate 500% increase in these rates. As a model substrate we took firefly luciferase, for which the unfolding and refolding rates are known (Herbst et al. 1997; Herbst et al. 1998) and for which we determined the thermal unfolding transition using circular dichroism spectroscopy. In addition, we tested a more stable hypothetical protein with a melting temperature between 37 and 45°C and an assumed 5000-fold increase in unfolding rate upon temperature up-shift from 25 to 45°C, with very similar results (data not shown).

Calculation of R to T ratio

R to T ratios were calculated by summing the quantities of $\text{DnaK} \cdot \text{ADP} \cdot \text{SD}$, $\text{DnaK} \cdot \text{ADP}$, $\text{DnaK} \cdot \text{ADP} \cdot \text{SD} \cdot \text{DnaJ}_2$, $\text{GrpE}_2 \cdot \text{DnaK} \cdot \text{ADP} \cdot \text{SD}$, $\text{GrpE}_2 \cdot \text{DnaK} \cdot \text{ADP}$, and $\text{DnaK} \cdot \text{ADP} \cdot \text{DnaJ}_2$ and dividing the result by the sum of $\text{DnaK} \cdot \text{ATP} \cdot \text{SD}$, $\text{DnaK} \cdot \text{ATP}$, $\text{DnaK} \cdot \text{ATP} \cdot \text{SD} \cdot \text{DnaJ}_2$, $\text{GrpE}_2 \cdot \text{DnaK} \cdot \text{ATP} \cdot \text{SD}$, and $\text{GrpE}_2 \cdot \text{DnaK} \cdot \text{ATP}$ for every 10 seconds of each simulation.

Fig. 2 Refolding probability and model validation. Squares represent experimental data of in vitro refolding of chemically denatured luciferase (data from (Mayer et al. 2000b)). Error bars represent standard deviations of independent experiments. Curve, simulation results with a refolding probability of 2%

Fig. 3 Curve fitting of reported temperature effects on reactions. Circles, reported experimental data (A–D from (Farr and Witt 1999), E and F from (Grimshaw et al. 2001)). Curves: fitting results. **(A)** Binding of substrate S^D to DnaK·ATP. **(B)** Dissociation of S^D from complexes with DnaK·ATP. **(C)** Binding of substrate S^D to DnaK·ADP. **(D)** Dissociation of S^D from complexes with DnaK·ADP. **(E)** Observed rate constants from R to T state conversion of DnaK. **(F)** Observed rate constants from T to R state conversion of DnaK. Fitting functions are given in Table 2

Simulation

The simulations were based on mass action processes. Because Gillespie's exact stochastic simulation algorithm (Gillespie 1976) limits the reactants of a reaction to less than 3, and in our model the reactions for R to T state

conversion of DnaK takes 3 reactants, we could use only ordinary differential equations to simulate the model. E-CELL v3, an open-source computer software package developed at Keio University for large-scale cellular event simulation (www.e-cell.org) (Tomita et al. 1999; Takahashi et al. 2004), was used to do all the simulations.

Table 2 Temperature dependence of reaction rates. Curve-fitting parameters from Fig. 3 and estimated effects of temperature on reaction rates between 25 and 45°C.

Reaction	Rate
S^N unfolding	$4.9 \cdot 10^{-5} \text{ s}^{-1}$ at 25°C; $4.5 \cdot 10^{-3} \text{ s}^{-1}$ at 45°C
$k_{\text{on}}^{\text{ATP}}$	$k = 4545.3 \cdot t + 2.6255 \cdot 10^5$
$k_{\text{off}}^{\text{ATP}}$	$k = 0.00011 \cdot t^4 - 0.0094333 \cdot t^3 + 0.34975 \cdot t^2 - 5.691 \cdot t + 35.889$
$k_{\text{on}}^{\text{ADP}}$	$k = 116.45 \cdot t^4 - 12385 \cdot t^3 + 4.992 \cdot 10^5 \cdot t^2 - 8.9154 \cdot 10^6 \cdot t + 5.9179 \cdot 10^7$
$k_{\text{off}}^{\text{ADP}}$	$k = 2.433 \cdot 10^{-7} \cdot t^4 - 2.3626 \cdot 10^{-5} \cdot t^3 + 0.00091419 \cdot t^2 - 0.015807 \cdot t + 0.10274$
R to T	$k = -1.732 \cdot 10^{-5} \cdot t^4 + 0.0017096 \cdot t^3 - 0.058399 \cdot t^2 - 0.97683 \cdot t - 5.2021$
T to R	$k = -1.2706 \cdot 10^{-7} \cdot t^4 + 2.2411 \cdot 10^{-5} \cdot t^3 - 0.00093831 \cdot t^2 + 0.015292 \cdot t - 0.077358$
Others	Increase to 5 times its value at 25°C

t, temperature in degrees Celsius; k, rate constant value

Results

Model construction

A computational model was developed as described in Materials and Methods (Fig. 1B and Table 1). The model was validated by simulating the refolding of 1000 molecules of denatured proteins at 25°C and then comparing the results to experimental data on the refolding of denatured firefly luciferase (Fig. 2). For the denatured protein (S^D), we made no assumptions about its nature other than that it was inactive and could be recognized as a substrate by DnaK and DnaJ. The denatured state could also be oligomeric. In contrast, the so-called aggregated state (S^A) in our model describes irreversibly aggregated molecules that cannot be rescued and refolded by the DnaK system alone in the absence of the Hsp100 chaperone, and that are therefore removed from the equilibria.

There are some differences in rate constants between this model and our previous model (Hu et al. 2006) because of differences in the parameters used for substrate binding and release in the R-to-T and T-to-R conversions. In our previous model, the parameters were selected for in vitro refolding of chemically denatured firefly luciferase, which was chosen as a model system in order to avoid temperature-induced changes in rate constants. Data for the effects of temperature on substrate binding and release are available for the interaction of DnaK with the peptide fVSV13 (representing amino acids 490–502 of the vesicular stomatitis virus glycoprotein) (Farr and Witt 1999) and were therefore used in the current model. However, these differences in the parameters are minor, as evidenced by the fact that the simulation results from the new model were very close to the results from the previous model and fit the experimental data equally well (Fig. 2). The determined refolding probability value, which gave the best fit to the data, was 2%—very close to the probability value used in our previous model (2.68%).

Implementing temperature effects on refolding kinetics

DnaK, DnaJ, and GrpE are affected differently by temperature, as reflected in the differences in the rate constants of the reactions involving the corresponding chaperone proteins. In particular, the rate of DnaJ-catalyzed T to R conversion shows an exponential increase with increasing temperature between 15 and 48°C, whereas in the same temperature range the GrpE-accelerated R to T conversion increases pro-

gressively less and even decreases when the temperature is raised above approximately 40°C (Grimshaw et al. 2001). On the basis of these findings, Christen and colleagues hypothesized that GrpE acts a thermosensor in the Hsp70 chaperone system by shifting DnaK towards its high-affinity R state at high temperatures (Grimshaw et al. 2001; Grimshaw et al. 2003). However, to understand how temperature affects the chaperone system, we have to consider all reactions happening during the refolding reaction cycle. The binding and release of a peptide substrate to DnaK in both the ADP and ATP states have been studied at different temperatures from 20 to 45°C (Farr and Witt 1999). The influence of temperature on the basal ATP hydrolysis rate has also been studied (Vogel et al. 2006). For other reactions (mainly those among chaperones), no data are available.

In Fig. 3, we show the results of curve-fitting of the reported temperature effect on each elementary reaction. To explore the behavior of the Hsp70 chaperone system in vitro at different temperatures, we compared three scenarios. In scenario 1 (the control scenario), temperature increases the protein-unfolding rate only and has no effects on any of the other reactions in the system. In scenario 2 (the wild-type scenario), temperature increases not only the unfolding rate but also the rates of all the other reactions in accordance with the curve-fitting functions (Fig. 3 and Materials and Methods). In scenario 3 (the GrpE mutant scenario), GrpE is considered thermostable without unfolding at the temperature under consideration. For this scenario only the first 4 data points in Fig. 3E were fitted by a polynomial function, thereby avoiding the rate decrease above 40°C. This function was used to extrapolate the rate of R to T conversion at 45°C. The refolding probabilities (2%) were identical in all simulations.

Temperature up-shift in vitro

First we asked how the Hsp70 system contributes to survival at elevated temperatures. We therefore started with 1000 molecules of the model protein in the native state at 25°C in the absence or presence of chaperones. At time-point 0 we raised the temperature to 45°C, which was simulated by the appropriate changes in reaction rates, and followed the fate of the model protein in the active state, the denatured state free in solution, and the aggregated state. In addition, we followed the R to T ratio of DnaK to monitor the relative effects of DnaJ and GrpE (Fig. 4). For comparison we followed protein molecules that were kept at 25°C.

Fig. 4 Temperature up-shift in vitro. A total of 1000 S^D molecules were added at time 0 with all the rate constants reflecting a temperature of 45°C. **(A)** Number of proteins remaining in the native state. **(B)** Number of proteins in aggregates. **(C)** Number of proteins in the unfolded state and free in solution. **(D)** R-to-T ratio. In all panels: Dashed black curve, scenario 1. Solid black curve, scenario 2. Dashed-dot black curve: scenario 3. Dotted black curve: no chaperone. Solid gray curve: simulation result with chaperone at 25°C. Dotted gray curve: simulation result in the absence of chaperone at 25°C. Note that scenario 3 is obscured by scenario 2 in panels A to C

At 25°C the protein was stable in the presence of chaperones but not in their absence, which is consistent with in vitro observations for firefly luciferase. At 45°C the number of active protein molecules decreased rapidly in the absence of chaperones, the number of denatured species increased equally rapidly but transiently, and the protein molecules were finally converted into the aggregated state in less than 2 h. In the presence of the Hsp70 system, the number of active proteins decreased less rapidly than in the absence of chaperones. Thereby, the Hsp70 system performed much better in maintaining active protein concentration when all the effects of temperature on the different reaction rates were included (scenario 2, Fig. 4A) than when the reaction rates were left constant at the values determined at low temperature (scenario 1, Fig. 4A). Surprisingly, we found only minor differences in the number of active (Fig. 4A), aggregated (Fig. 4B), or denatured proteins (Fig. 4C) when GrpE was modeled to be thermostable (scenario 3), i.e. with increased rates at 45°C instead of the in vitro observed decreased rates (scenario 2), although we found, as predicted, a clear difference in the R to T ratio between these two scenarios (Fig. 4D). Taken together, our simulation results demonstrate that the temperature-induced differential increase in the individual reaction rates of the Hsp70 system favors a slower loss of active protein species compared with the computer modeled GrpE thermostable mutant.

Transient heat shock in vitro

To explore how temperature-induced changes in reaction rates influenced recovery after the return to optimal temperature conditions, we simulated a transient heat shock by increasing the appropriate reaction rates over 10 and 30 min and then setting them back to their original values. Figure 5A shows that in scenario one (dashed line) the proportion of native proteins decreased rapidly to about 35% during the 10-min heat shock (identical to the result in Fig. 4A), and then recovered to about 85% after return to 25°C due to chaperone-mediated refolding. When the effects of temperature on all the reactions of the chaperone cycle were considered (scenario 2, solid line in Fig. 5A), the concentration of native proteins decreased less dramatically upon temperature up-shift, reaching a value of about 56% during the 10-min heat-shock period. Interestingly, during the recovery phase the number of proteins refolded by the chaperone system was lower than in scenario 1, and the proportion of native proteins reached about 76% of that before the heat shock (Fig. 5A). Scenario 3 was again of only minor difference to scenario 2, indicating that under our conditions GrpE was not limiting for the system. However, the R to T ratio was clearly influenced by the different activities of GrpE under the two scenarios (Fig. 5D).

In tracking the protein aggregates under the three scenarios, we observed that more aggregates were formed under scenarios 2 and 3 than under scenario 1

Fig. 5 Transient heat shock in vitro. A transient heat shock was simulated by adding 1000 folded S^N proteins at 25°C and increasing the appropriate reaction rate constants for 10 min. **(A)** Number of proteins remaining in the native state. **(B)** Number of proteins in aggregates. **(C)** number of proteins unfolded, free in solution. **(D)** R-to-T ratio. Legends for curves are the same as in Fig. 4. Note that scenario 3 is obscured by scenario 2 in panels A to C

(Fig. 5B). It is interesting that the concentration of free denatured protein resulting from heat-induced unfolding was tightly controlled when the temperature effect on the chaperone actions was taken into account. The concentration of denatured proteins in scenarios 2 and 3 was less than half of that in scenario 1 (Fig. 5C). The results of the 30-min transient heat shock were similar to those of the 10 min heat shock (data not shown).

Thus we conclude that the intrinsic temperature effects on the Hsp70 chaperone system increase the level of functional proteins and a decrease levels of denatured proteins during heat shock. However, this advantage is achieved at the cost of increased protein aggregation.

Temperature up-shift in vivo

To explore how temperature shift may effect protein recovery in the Hsp70 system, we simulated a 10-min heat shock and tripled the levels of DnaK, DnaJ, and GrpE at 3, 5, and 10 min after temperature up-shift. Although our model, in accordance with the optimal concentrations for in-vitro refolding, already contained 10, 2, and 5 times more DnaK, DnaJ and GrpE, respectively, than the model protein, a further, 300%, increase in the concentrations of chaperones immediately led to a dramatic increase in the proportion of active protein (Fig. 6A) and a decrease in

free denatured (Fig. 6C) and aggregated species (Fig. 6B). After the end of the heat shock, refolding was also more efficient when the chaperone levels were increased, and a higher final yield of reactivated proteins was achieved (Fig. 6A). Even an increase in chaperone levels after 10 min, concomitant with the end of heat shock, still had a beneficial effect on the final yield of refolded proteins (Fig. 6A). The timing of chaperone level increase had a clear effect on the final yields of active and aggregated proteins but not on the levels of denatured proteins, which were not in any chaperone complexes. Increasing the chaperone levels at 3 or 5 min after temperature up-shift led to the same final level of free denatured proteins; the 5-min curve was simply shifted to the right (Fig. 6C). The level of free denatured proteins paralleled the R to T ratio: the lower the R to T ratio the lower the level of free, denatured proteins (Fig. 6C and D). Taken together, our heat shock response experiments clearly indicate that despite the presence of an already large excess of chaperones over model protein at the beginning of the experiment, a further increase in chaperone levels increased the levels of active protein during heat shock and the yield of reactivated protein after the end of heat shock. At the same time, the level of denatured proteins free in solution was directly correlated with the R to T ratio of DnaK and therefore with the relative activities of DnaJ and GrpE.

Fig. 6 Temperature up-shift in vivo. A transient heat shock was simulated as in Fig. 5, but with a 3-fold increase in the quantity of DnaK, DnaJ, and GrpE at 3 (solid curve), 5 (dashed curve) or 10 min (dotted curve) after the start of transient heat shock. Gray dashed curve, no heat shock (25°C). Gray solid curve, no increase in the quantities of DnaK, DnaJ, and GrpE. HS indicates the duration of the transient heat shock. **(A)** Number of proteins remaining in the native state. **(B)** Number of proteins aggregated. **(C)** Number of proteins in the unfolded state, free in solution. **(D)** Dynamics of R-to-T ratio

Discussion

We investigated the consequences of temperature dependence of the individual reaction rates of the Hsp70 system on the maintenance of active protein during heat shock; the prevention of aggregation; the level of denatured protein species free in solution; and recovery after the end of heat shock. Since different dependencies of the individual rates on temperature are possible, as exemplified by the thermolability of GrpE, it is conceivable that the observed dependence on temperature is a product of an evolutionary optimization process. Our simulation data suggest that in *E. coli* the naturally evolved chaperone system maintains the concentration of active protein as high as possible during heat shock, even at the cost of lower recovered activity after return to optimal growth conditions.

We did not expect that the computer-modeled thermostable GrpE mutant in scenario 3 would show no disadvantage when compared with the wild-type GrpE (scenario 2) in both the in vitro temperature up-shift simulation (Fig. 4A–C) and transient heat shock simulation (Fig. 5A–C). This showed that, in our simulation setting, the R to T state transition accelerated by GrpE is not rate limiting. When the R to T transition becomes rate limiting, the advantage of naturally evolved GrpE in protecting cellular proteins may become clear. When multiple DnaKs can help a single substrate to fold, then R to T conversion may be more important than it appears in our model and

scenario 2 may perform significantly better than scenario 3.

The GrpE- and DnaJ-controlled R to T ratio is considered a key point in the regulation of the Hsp70 chaperone system (Grimshaw et al. 2001). However, real-time recording of the dynamics of the R to T ratio is difficult to perform using current laboratory facilities. Figure 5D is an interesting result of real-time R to T ratio dynamics from our computer simulation of the chaperone system action during a transient heat shock response. With the start of heat shock (time 0), in all three scenarios the R to T ratio increased rapidly, reflecting the accumulation of unfolded proteins, which were rapidly bound to DnaK and induced ATP hydrolysis (Fig. 5C). The R state was slowly converted to the T state until the end of heat shock in scenario 2 and 3, which is in contrast to the continuing increase in the R to T ratio in scenario 1. This suggests that at high temperatures it becomes more difficult for newly unfolded proteins to enter the chaperone cycle, because if the chaperone could enter the cycle at the same rate of refolding, a plateau should have existed before the end of heat shock. The peaks in the R to T ratio immediately following transient heat shock shows that 25°C is much more favorable than 45°C for chaperone system function, and that substrate proteins can finish the chaperone cycle much faster at this temperature in all the three scenarios (compare the decrease in R to T ratio in Figs. 4D and 5D). The R to T ratio change clearly reveals that a huge amount of potential energy is stored in the DnaK chaperone

system. The purpose of storing such potential energy could be to prevent substrate release under unfavorable conditions when the substrate cannot fold back into the native state. It could also prevent futile ATPase cycles of the chaperone system, which is important given that during heat shock the Hsp70 system needs to protect a large number of proteins inside the living cell while the ATP production machinery is compromised. As we have shown in our previous work (Hu et al. 2006), ATP consumption by the Hsp70 system depends on the folding status of the substrate protein, expressed as refolding probability per Hsp70 cycle. In such a setting, the R to T ratio changes in scenario 2 can lower ATP consumption considerably.

In this work we could validate only our simulation result at 25°C (Fig. 2) by comparison with experimental results. To validate the simulation results of scenarios 2 and 3, wild-type chaperones and thermostable GrpE mutants, respectively, can be used. For example, Grimshaw et al. introduced an intersubunit disulfide bond into the paired long helices of the GrpE dimer (Grimshaw et al. 2003). With disulfide-stabilized GrpE (GrpE R40C), the rate of ADP/ATP exchange and conversion of DnaK from the R state to the T state increased continuously with increasing temperature; this is also suggested by our simulation result (Fig. 4D).

There are several limitations to our model. One of the major limitations is that our model did not include the time delay for unfolded GrpE to refold and restore its activity, because so far no such information has been published. We also assumed that only one DnaK was involved in one chaperone cycle. In our previous work (Hu et al. 2006), we discussed that it might be possible for multiple DnaKs to be working simultaneously in the chaperone cycle, but direct experimental results are still lacking. If multiple DnaKs indeed work together, the naturally evolved GrpE thermal characteristic may have more impact on chaperone regulation. The possibility of multiple DnaKs working simultaneously on a single substrate shall be addressed in future work using both experiments and computer simulation.

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