GroES binding regulates GroEL chaperonin activity under heat shock

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Abstract Chaperonins $GroEL_{14}$ and $GroES_7$ are heat-shock proteins implicated in the molecular response to stress. Protein fluorescence, crosslinking and kinetic analysis revealed that the bond between the two otherwise thermoresistant oligomers is regulated by temperature. As temperature increased, the affinity of $GroES_7$ and the release of bound proteins from the chaperonin concomitantly decreased. After heat shock, $GroES_7$ rebinding to $GroEL_{14}$ and $GroEL_{14}GroES_7$ particles correlated with the restoration of optimal protein folding/release activity. Chaperonins thus behave as a molecular thermometer which can inhibit the release of aggregation-prone proteins during heat shock and restore protein folding and release after heat shock.

Key words: GroE chaperone; Molecular thermometer; Heat shock; Protein folding

1. Introduction

Heat-shock proteins GroEL and GroES from *Escherichia coli* (*E. coli*) belong to a ubiquitous class of molecular chaperonins, implicated in the folding of de novo synthesized and stress-denatured proteins (for a review, see [1,2]). In vitro, the $GroEL_{14}$ core oligomer (L_{14}) can spontaneously bind non-native proteins and prevent protein aggregation [3,4]. Under conditions where there is no spontaneous protein refolding, ATP hydrolysis and the presence of co-chaperonin $GroES_7$ (S_7) are essential for rapid and efficient folding of the bound proteins [3,5].

Central to an understanding of the chaperonin mechanism under physiological and stress conditions is the nature of the various GroEL-GroES hetero-oligomers involved in the protein folding cycle. In the presence of high ADP, low S_7, Mg^{2+}, or ATP concentrations, only one S_7 may bind one end of the L_{14} cylinder and form an asymmetric $L_{14}S_7$ particle [6–10]. ATP hydrolysis accelerates binding and release of S_7 and the folding of the bound protein within the central cavity of L_{14} [11–13]. However, under physiological pH, Mg^{2+} and chaperonin concentrations, a second S_7 can bind $L_{14}S_7$ and form a symmetric $L_{14}(S_7)_2$ complex [7–10,14–16], whose relative amount in the solution correlates with the rate and efficiency of the protein-folding reaction [10,15,17,18].

We addressed here the effect of heat-shock temperatures on the steady-state distribution of chaperonin oligomers L_{14}, $L_{14}S_7$ and $L_{14}(S_7)_2$, and on the ATPase and protein folding activity. We found that the temperature-dependent affinity of S_7 for L_{14} and $L_{14}S_7$ regulates chaperonin activity during and after heat shock in a biologically relevant manner.

2. Materials and methods

2.1. Materials

$GroES_7$ and $GroEL_{14}$ oligomers were purified as in [19]. Chaperonin concentrations were expressed as protomers. Porcine mitochondrial malate dehydrogenase (MDH) and muscle lactate dehydrogenase (LDH) were from Boehringer Mannheim, pyruvate kinase from Sigma.

2.2. Chaperonin activity

Chaperonin-mediated protein folding activity was performed in 50 mM triethanolamine, 5 mM dithiothreitol, 20 mM $MgAc_2$, 20 mM KCl, 3 mM phosphoenol pyruvate and 20 μg/ml pyruvate kinase (buffer A) and ATP as specified. The ATP regeneration was fully active after 30 min at 47°C (not shown). Thermal denaturation of MDH (0.25 μM) in buffer A was for 20 min at 47°C in the presence or absence of GroEL (3.5 μM), GroES (0–56 μM) and ATP as specified. The chaperonin-assisted MDH refolding was initiated by downshifting the temperature to 25°C and ATP addition.

Denaturation of LDH (25 μM) was in 5 M urea and 10 mM dithiothreitol for 5 min at 0°C, followed by a 100-fold dilution in buffer A containing chaperonins as specified. LDH refolding was initiated at 25°C or 47°C with 1 mM ATP. Rates of LDH refolding were extracted from the linear phase of the refolding reaction, between 4 and 14 min for both temperatures (not shown). After 20 min at 25°C, the maximal yield of recovered LDH was 33% of native control, a tenth of which was produced by spontaneous refolding. At 47°C, the yield was 12%, without spontaneous refolding.

2.3. Enzymatic activities

The activity of MDH and LDH was measured as in [18] and [20], respectively. The GroEL ATPase was measured as in [21]. At indicated temperatures and GroES concentrations, initial rates of ATPase were measured in the presence of 1 mM ATP, which was saturating at all temperatures measured (not shown).

2.4. AEDANS labeling and fluorescence measurements

$GroEL_{14}$ was labeled with 5-((((2-iodoacetyl)amino)ethyl)amino)-naphthalene-1-sulfonic acid (AEDANS) (Molecular Probes Europe BV) and the fluorescence of AEDANS-L_{14} was measured at indicated temperatures as in [10]. Structurally and enzymatically, AEDANS-L_{14} was as thermostable as L_{14} (not shown).

2.5. ^3H-labeling and protein crosslinking

Native MDH was labeled with $NaB(^3H)_4$ as described for GroES in [22]. [^{3}H]MDH-chaperonin complexes were fully crosslinked with glutaraldehyde as in [7,23,24]. MDH binding was inferred from the total ^3H label in the 900–500 kDa fraction after gel filtration on a Superose 6B column (Pharmacia). Partial crosslinking of transient L_7 and L_7S_7 species (Fig. 2B) was first carried out for 30 s at the indicated temperatures, then for 7 min at 37°C. Crosslinked complexes were then separated from unbound MDH by SDS-gel electrophoresis as in [15,23,24].

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Abbreviations: MDH, mitochondrial malate dehydrogenase; LDH, lactate dehydrogenase; S:L, GroES:GroEL molar ratio; L_{14}, $GroEL_{14}$; S_7, $GroES_7$

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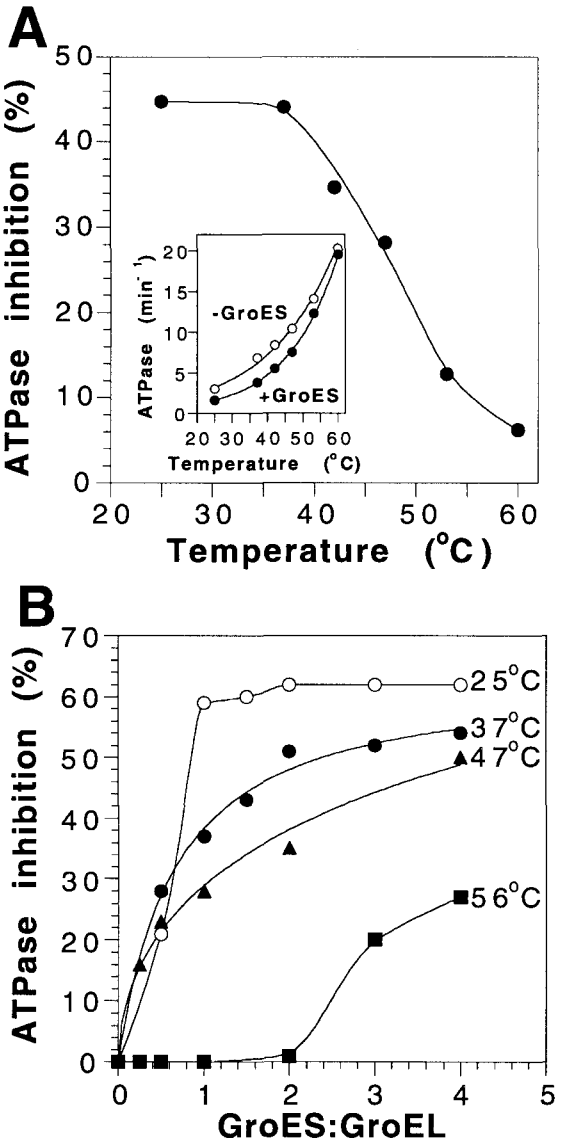

Fig. 1. Effect of temperature on the GroEL ATPase activity. A: Temperature-dependent inhibition of the GroEL (3.5 μM) ATPase by GroES (5.25 μM). Inset: Temperature-dependent ATPase of GroEL, with (●) or without (○) GroES. B: GroES-dependent inhibition of the GroEL ATPase at various temperatures.

3. Results

L_{14} is a thermoresistant oligomer, whose ATPase activity increased with temperature (Fig. 1A, inset), as also shown previously [25]. However, the ability of GroES to inhibit the GroEL ATPase decreased as the temperature increased (Fig. 1A). Hence, the maximal ATPase inhibition by GroES at 25°C was reduced by half at 47°C (Fig. 1A). The original GroES inhibition levels were recovered after the heat shock (not shown), showing that the effect of temperature was fully reversible. GroES inhibition could be restored at high temperature by increasing the S:L ratio above the physiological 1:1 ratio [26] (Fig. 1B). At 47°C, about 2.5 times more GroES was required to cause half of the maximal GroES inhibition than at 25°C. Thus, GroES was fully functional at 47°C, but with a decreased affinity for GroEL. The thermal sensitivity of GroES binding to GroEL was observed directly by crosslinking analysis. In the presence of S:L = 1.5 and saturating ATP, the amount of transient crosslinking species L_7S_7 decreased at the expense of L_7 as the temperature rose (Fig. 2A). A majority of $L_{14}(S_7)_2$ particles ($L_7S_7 > > L_7$, see [15]) at 37°C became a majority of $L_{14}S_7$ ($L_7S_7 = L_7$) at 47°C, which returned to be a majority of $L_{14}(S_7)_2$ seconds after downshifting the temperature back to 37°C.

Fluorescence analysis of AEDANS-L_{14} can discriminate between the binding of a single S_7 to L_{14} and the binding of a second S_7 to $L_{14}S_7$ [10]. Analysis of the GroES titration curves (representative curves: Fig. 2B, inset) allowed the derivation of two apparent S_7 binding constants: EC_{25} for $L_{14}+S_7 \leftrightarrow L_{14}S_7$, and EC_{75} for $L_{14}S_7+S_7 \leftrightarrow L_{14}(S_7)_2$. At temperatures below 39°C, the affinity of S_7 for L_{14} ($1/EC_{25}$) remained 3–5-fold higher than for $L_{14}S_7$ ($1/EC_{75}$), confirming the negative cooperativity of S_7 binding between the two L_{14} rings [7,11]. Above 39°C, however, the affinity of S_7 for $L_{14}S_7$ decreased sharply, as compared to L_{14} (Fig. 2B). Thus, the decrease in S_7 affinity during heat shock primarily affected the stability of the $L_{14}(S_7)_2$ species.

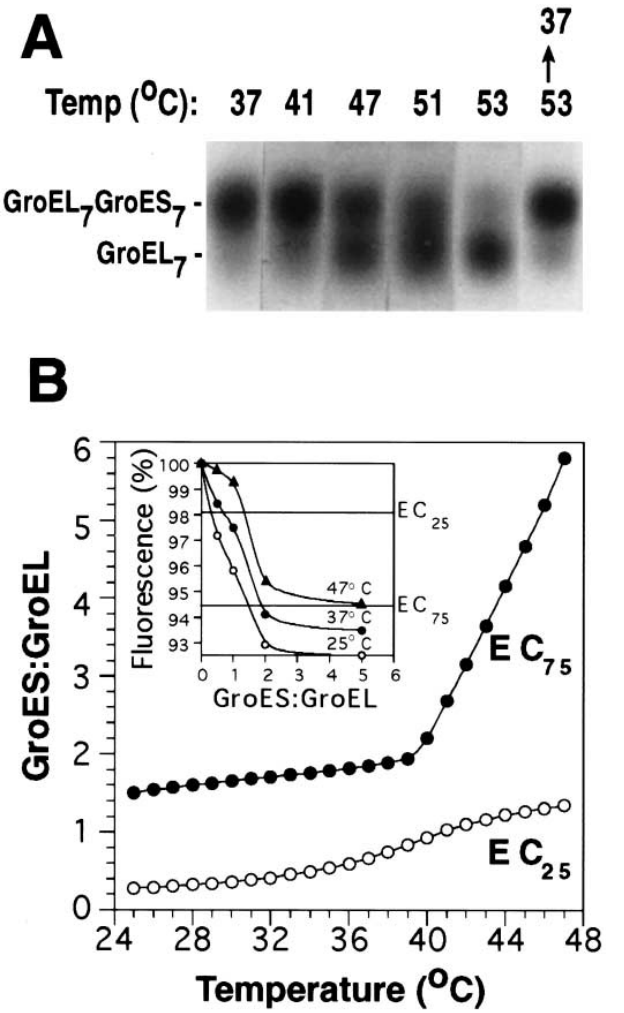

Fig. 2. Effect of temperature on the affinity of GroES. A: Distribution of partial crosslinking species; L_7 and L_7S_7 [15] under conditions as in Fig. 1A. B: Fluorescence-derived apparent binding S_7 constants for $S_7+L_{14} \leftrightarrow L_{14}S_7$ (EC_{25}) (○) and $S_7+L_{14}S_7 \leftrightarrow L_{14}(S_7)_2$ (EC_{75}) (●), at increasing temperatures. Inset: The effect of GroES on the relative fluorescence intensity (%) of AEDANS-L_{14} (representative curves), at increasing temperatures.

Next, we addressed the effect of temperature-dependent changes in GroES affinity and stability of $L_{14}(S_7)_2$ in particular, on the chaperonin activity during and after heat shock. When native MDH was exposed to 47°C, it was inactivated at a rate of ~0.13/min, with or without chaperonins and ATP (Fig. 3A). After heat shock, a strict ATP- and GroES-dependent refolding of MDH was observed, provided L_{14} was present during the heat denaturation. Remarkably, similar high yields of MDH recovery (~60%) were obtained regardless of whether ATP was present during or added after the heat shock (Fig. 3A).

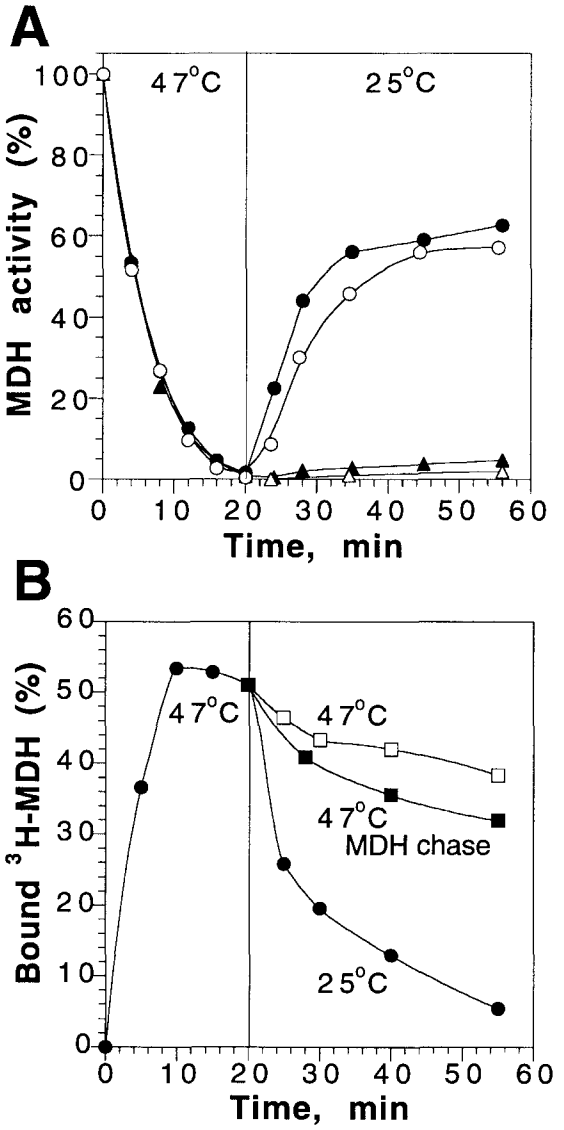

Fig. 3. Chaperonin-mediated protein-folding activity during and after heat shock. A: Temperature-dependent inactivation and reactivation of MDH. MDH activity (0.25 μM) was measured during 20 min at 47°C (HS), then during 36 min at 25°C, in the presence of GroEL (3.5 μM), GroES (5.25 μM) and ATP (0.5 mM) during HS (●); GroEL, GroES during HS, ATP after HS (○); GroES, ATP during HS, GroEL after HS (▲); GroES during HS, ATP after HS (△). B: Temperature-dependent binding and release of MDH to and from chaperonins. Native [^{3}H]MDH was incubated during HS with GroEL, ATP and GroES as in A. Then, the temperature was either decreased to 25°C (●), or kept for another 36 min at 47°C in the absence (□) or presence (■) of a 3-fold excess (0.75 μM) of unlabeled MDH.

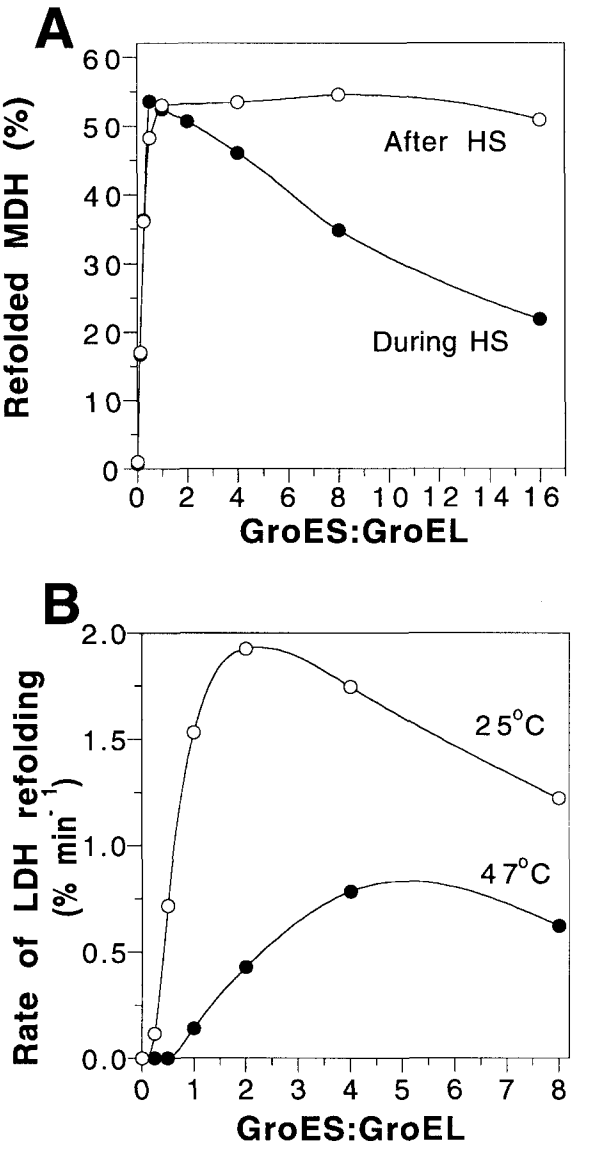

Fig. 4. A: GroES-dependent irreversible MDH inactivation during heat shock. MDH was incubated with GroEL, ATP at 47°C. Increasing GroES concentrations were present either during (●) or after (○) heat shock. Maximum yields of MDH activity were measured 60 min after the transfer to 25°C. B: Rate of GroES-dependent GroEL-assisted refolding of LDH at 25°C (○) or 47°C (●). Rates of LDH refolding are expressed as the fraction (%) of refolded LDH per min, as compared to a non-denatured LDH control.

The ability of chaperonins to bind and release bound MDH at 47°C and 25°C in the presence of S_7 and ATP was addressed by gel separation of the crosslinked [^{3}H]MDH-chaperonin species from free [^{3}H]MDH (Fig. 3B). During heat shock, denatured ^{3}H-labeled MDH bound the chaperonin at an initial rate of ~0.08/min (Fig. 3B). Binding was saturated within 10 min with about 0.6 MDH molecule per L_{14} oligomer, in agreement with the maximal yields of recovered MDH after the heat shock (Fig. 3A). A net slow protein release followed at 47°C (~0.01/min), without recovery of enzymatic activity (not shown). When, after 20 min at 47°C, a 3-fold excess of unlabeled native MDH was added, a minor 1.5-fold increase in the slow rate of non-productive protein release was

observed. When the temperature was down-shifted to 25°C, the rate of protein-release sharply increased 5.5-fold (~0.05/min) (Fig. 3B) and active MDH was recovered (as in Fig. 3A).

The amount of active MDH recovered after the heat shock was optimal when the S:L ratio during the heat shock was 1:1 as in the cell (Fig. 4A). However, when the GroES concentration during the heat shock exceeded that of GroEL, the yield of MDH recovered after the heat shock decreased proportionally to the GroES concentration. Remarkably, excess GroES was deleterious to MDH recovery only when present together with ATP during, not after the heat shock (Fig. 4A).

In contrast to thermolabile MDH, excess GroES during heat shock activated the chaperonin refolding of the thermostable enzyme LDH, up to S:L = 4. Corroborating the ATPase-derived decrease in the GroES affinity for GroEL (Fig. 1B), half of the maximal rates of LDH refolding were observed at S:L = 0.65 and 1.90, at 25°C and 47°C, respectively (Fig. 4B). Thus, although chaperonins could actively fold thermostable LDH at 47°C, at S:L = 1 as in the cell, the rate of LDH folding was suboptimal as compared to 25°C, because of the decreased affinity of S_7.

4. Discussion

It has been previously shown by circular dichroism [27], fluorescence, light scattering [28] and refolding activity assays [29] that $GroEL_{14}$ and $GroES_7$ are thermoresistant, up to 60°C. While this was confirmed here, and further demonstrated by crosslinking (Fig. 2A), the ATPase activity and AEDANS fluorescence indicated that the bond between S_7 and L_{14} or $L_{14}S_7$ oligomers was thermolabile, though in a fully reversible manner (Figs. 1 and 2B). Crosslinking and fluorescence showed that the $L_{14}(S_7)_2$ hetero-oligomer was particularly unstable above 39°C, as compared to $L_{14}S_7$ (Fig. 2). In contrast to 37°C, where chaperonins and ATP did protect or reactivate some of the denaturing MDH [30], MDH was denatured at the same rate without GroEL, GroES and ATP under heat-shock temperatures (47°C) (Fig. 3A). The observation that chaperonins and ATP during the heat shock did not reduce the yields of MDH (Fig. 3A) recovered after the heat shock further indicates that during heat shock, the protein folding/release mechanism of chaperonins is transiently inhibited. This was directly shown by MDH crosslinking with chaperonins and gel filtration (Fig. 3B). Under the same conditions where chaperonin hetero-oligomers were found to redistribute into a majority of $L_{14}S_7$ at 47°C, as compared to $L_{14}(S_7)_2 > L_{14}S_7$ at 25°C (Fig. 2), the rate of protein release was 5.5 times lower at 47°C than at 25°C, despite the 5 times higher ATPase activity (Fig. 1A).

Protein release could be artificially induced at high temperature in the presence of a non-physiological excess of GroES over GroEL. In the case of thermolabile MDH (Fig. 4A), aspartate aminotransferase [29] and RNA polymerase [31], excess GroES and protein release during heat shock led to irreversible inactivation. In the case of thermostable LDH (Fig. 4B) or Fab fragments [32], excess GroES and protein release during heat shock led to active protein refolding and reactivation. Thus, at high temperature, chaperonins are fully able to fold proteins (Figs. 1 and 4B), provided the proteins can remain folded, and that excess of GroES can compensate for the decreased affinity of GroES for GroEL. However, at equimolar GroES and GroEL as in the cell, increasing temperature reversibly reduced both the amount of $L_{14}(S_7)_2$ species in the solution and the protein folding/release activity. Since the $L_{14}(S_7)_2$ species correlates with rapid and efficient protein refolding activity [15,18], this suggests that the thermolabile $L_{14}(S_7)_2$ species may serve as a molecular thermometer that can regulate chaperonin protein folding/release activity during and after heat shock.

The rate of a natural heat shock, such as the rising of the sun, can be closely followed by a significant increase in the cellular amounts of chaperones [1,26]. Moreover, the interaction with other chaperones may further extend the contribution of GroE chaperonins to the cellular response to heat stress. Hence, Hsp25 can serve as a surrogate protein-binding chaperone to DnaK/DnaJ/GrpE [33], which, in turn, can serve as a protein-binding surrogate to GroESL [34,35]. However, GroESL chaperonins preferentially bind and fold later folding intermediates, such as molten globules [2,35], and may therefore thermoregulate the last steps of a multi-chaperone machinery in the cell.

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