Temperature-Controlled Activity of DnaK−DnaJ−GrpE Chaperones: Protein-Folding Arrest and Recovery during and after Heat Shock Depends on the Substrate Protein and the GrpE Concentration†

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ABSTRACT: Heat-shock proteins DnaK, DnaJ, and GrpE (KJE) from *Escherichia coli* constitute a three-component chaperone system that prevents aggregation of denatured proteins and assists the refolding of proteins in an ATP-dependent manner. We found that the rate of KJE-mediated refolding of heat- and chemically denatured proteins is decreased at high temperatures. The efficiency and reversibility of protein-folding arrest during and after heat shock depended on the stability of the complex between KJE and the denatured proteins. Whereas a thermostable protein was released and partially refolded during heat shock, a thermolabile protein remained bound to the chaperone. The apparent affinity of GrpE and DnaJ for DnaK was decreased at high temperatures, thereby decreasing futile consumption of ATP during folding arrest. The coupling of ATP hydrolysis and protein folding was restored after the stress. This strongly indicates that KJE chaperones are heat-regulated heat-shock proteins which can specifically arrest the folding of aggregation-prone proteins during stress and preferentially resume refolding under conditions that allow individual proteins to reach and maintain a stable native conformation.

The heat-shock protein Hsp70 belongs to a ubiquitous family of molecular chaperones found in the cytosol of prokaryotes and in various compartments of the eukaryotic cell (*1*, *2*). Under physiological conditions, members of the Hsp70 family participate in essential cellular functions, such as folding of nascent polypeptides, assembly of multimeric protein structures, membrane translocation of secreted proteins, signal transduction, and protein degradation (*3*−*5*). One of the Hsp70 proteins of *Escherichia coli*, DnaK (70 kDa), is cotranscribed with DnaJ (41 kDa) and GrpE (23 kDa), suggesting that all three cochaperones are a functional unit (*6*−*8*). Although the proteins are encoded on the same operon, the cellular concentrations of DnaK, DnaJ, and GrpE may vary. In nonstressed *E. coli* cells, there are estimated concentrations of 5 μM DnaK (*9*, *10*), 1 μM DnaJ (*9*, *11*) and 1−2 μM GrpE (*12*). This molar ratio of 5:1:1 will be described here as a physiological ratio of KJE.[1]

Like other Hsp70s, DnaK is a molecular chaperone that can bind denatured proteins and peptides in an extended conformation (*13*, *14*). Following ATP-coordinated cycles of binding and release, DnaK assists the refolding of denatured polypeptides into active proteins (*3*, *15*). Cochaperones DnaJ and GrpE separately and synergistically stimulate the ATPase activity of DnaK and thus increase the rate protein folding and release from DnaK (*16*). DnaJ can also bind and transfer non-native proteins to DnaK (*17*). Whereas GrpE is primarily considered a nucleotide exchanger (*18*), there is evidence that both DnaJ and GrpE can also directly affect the affinity of non-native proteins for DnaK (*19*, *20*).

Many Hsp70s, including DnaK, are stress-induced proteins essential to the survival and acclimation of prokaryotic and eukaryotic cells to environmental stresses (*21*). The fact that the cellular concentrations of DnaK increase 13-fold during a mild heatshock (*12*) adds to the genetic evidence that DnaK, DnaJ, and GrpE carry out functions essential to cell survival during heat shock. A majority of in vitro studies addressed the mechanism of the KJE chaperone at physiological temperatures (*11*, *15*, *19*, *22*−*24*). Here we addressed the molecular mechanism of KJE proteins, as an ATPase and a protein-folding chaperone system, under heat-shock and non-heat-shock conditions. The chaperone activity of the KJE system was found to be regulated by temperature; during heat shock, the refolding by KJE of some protein substrates was arrested, but was fully restored after the stress. The efficiency of protein-folding arrest during stress, and of protein recovery after the stress, depended on the nature of the protein substrate and on the GrpE concentration.

MATERIALS AND METHODS

Proteins. DnaK, DnaJ, and GrpE proteins were purified as described previously (*25*, *26*). All protein concentrations were expressed as molar concentrations of protomers and not of oligomers. Hog muscle lactate dehydrogenase (LDH) and porcine mitochondrial malate dehydrogenase (MDH) were from Boehringer-Mannheim. *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (G6P-DH) and rabbit muscle pyruvate kinase (PK) were from Sigma. Inorganic phosphorus was quantified using the Diagnostic Phosphorus Reagent from Sigma.

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[1] Abbreviations: KJE, DnaK−DnaJ−GrpE; MDH, malate dehydrogenase; LDH, lactate dehydrogenase; G6P-DH, glucose-6-phosphate dehydrogenase; PK, pyruvate kinase; TEA, triethanolamine; DTT, dithiothreitol.

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The stability of the protein substrates MDH, G6P-DH, and LDH was addressed by measuring the kinetics of the enzyme inactivation at various increasing temperatures. Native LDH (0.5 μM), MDH (0.25 μM), or G6P-DH (0.25 μM) was incubated at the indicated temperatures for increasing periods of time in 50 mM triethanolamine/HCl (TEA, pH 7.5), 20 mM $MgAc_2$, 150 mM KCl, and 5 mM dithiothreitol (DTT), without chaperones and nucleotides. Rates of enzyme inactivation were derived from the time curves, fitted to an exponential decay equation.

Enzyme Denaturation. Due to the various degrees of thermal stability between native MDH, LDH, and G6P-DH, each required different denaturation protocols to become substrates of the chaperone-mediated refolding reaction. Thermostable enzymes such as LDH and G6P-DH required an initial step of denaturation in urea, prior to dilution in the presence of chaperones. In contrast, heat-labile MDH was directly denatured by heat alone (47 °C) in the presence of the chaperones. (i) Native G6P-DH (12.5 μM) was first denatured in 5 M urea, 50 mM TEA (pH 7.5), and 10 mM DTT for 5 min at 47 °C and then diluted 50-fold (0.25 μM) into the folding buffer [50 mM TEA (pH 7.5), 20 mM $MgAc_2$, 150 mM KCl, 5 mM DTT, 0.1 mM ADP, and 3 mM phosphoenolpyruvate] containing DnaK, DnaJ, and GrpE as indicated. The mixture was further incubated for 15 min at 47 °C, prior to the initiation of refolding with ATP and PK. (ii) Native LDH (37.5 μM) was first denatured in 5 M urea for 30 min at 25 °C and then diluted 75-fold (to 0.5 μM) in the folding buffer containing DnaK, DnaJ, and GrpE as indicated. (iii) Native MDH (0.7 μM final concentration) was directly denatured for 30 min at 47 °C in the folding buffer (see above) containing DnaK, DnaJ, and GrpE as indicated.

Chaperone Activity. The initial concentrations of substrate enzymes were chosen so they were close to saturative with regard to chaperone binding, to obtain optimal rates, rather than optimal yields, of protein refolding. Thus, the refolding activity was expressed as a concentration (nanomolar) of recovered enzyme, per time unit, rather than as a fraction (%) of activity of the total native enzyme prior to denaturation. Refolding of LDH, G6P-DH, and MDH was initiated at the indicated temperatures upon addition of ATP (3 mM) and PK (20 μg/mL). Maximal rates of KJE-mediated refolding of MDH, LDH, or G6P-DH were derived from the linear phase of the time curves of recovered enzymatic activities (as in Figure 1 insets).

Enzymatic Activities. The assays for LDH and MDH activity were as described by Goloubinoff et al. (*27*). The G6P-DH activity assay was based on that of Hansen and Gafni (*28*), using 2.5 mM glucose 6-phosphate and 1 mM NAD. At the indicated temperatures and chaperone concentrations, the ATPase activity was measured (at 25 °C) in folding buffer containing 3 mM ATP and 20 μg/mL PK, using the colorimetric method for phosphate determination from Sigma, as in Mendoza et al. (*29*).

Calculations. In the presence of a saturative ATP concentration (3 mM), the activation of the DnaK ATPase by DnaJ, or GrpE, can reflect the extent of interaction between DnaJ, or GrpE, and DnaK. K_{Aapp} was the effective concentration of DnaJ, or GrpE, that induced half of the maximal activation of the DnaK ATPase in the presence of a saturative concentration of GrpE (DnaK−GrpE) or of DnaJ (DnaK−DnaJ), respectively. K_{Aapp} was derived from dose−response curves of DnaJ or GrpE, transformed to a double-reciprocal plot, after subtraction of the ATPase activities of the DnaK−GrpE and DnaK−DnaJ without DnaJ and GrpE, respectively. $1/K_{Aapp}$ was the apparent affinity of the cochaperones.

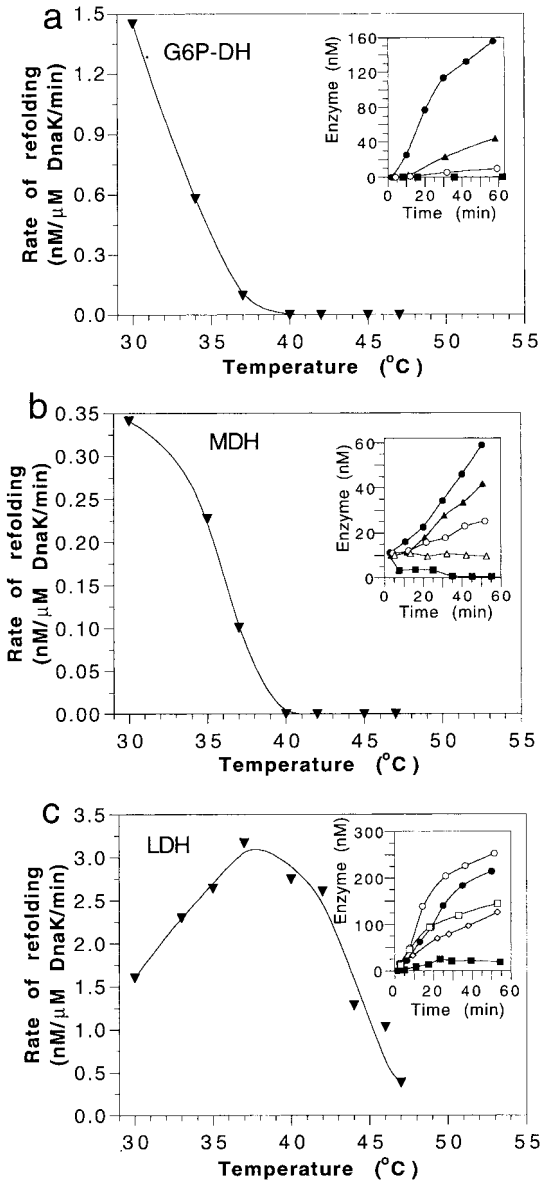

FIGURE 1: Effect of temperature on KJE-mediated refolding of proteins. (a) Urea- and heat-denatured G6P-DH, (b) heat-denatured MDH, or (c) urea-denatured LDH interacted with 3.5 μM DnaK, 0.7 μM DnaJ, and 0.35 μM GrpE in refolding buffer. Protein refolding at the indicated temperatures was initiated by addition of ATP, and the time-dependent reactivation was measured. Rates of KJE-mediated refolding were derived from time curves of enzyme reactivation. (Insets) Representative time curves at 30 °C (●), 34−35 °C (▲), 37 °C (○), 40 °C (△), 42 °C (□), 44 °C (◇), and 47 °C (■).

RESULTS

Temperature Controls KJE-Mediated Protein-Folding Activity. The refolding activity of the KJE chaperone system was examined at various temperatures, using three folding substrates: urea- and heat-denatured G6P-DH, heat-denatured MDH, and urea-denatured LDH (see Materials and Methods). We found that, in the presence of ATP, all three denatured

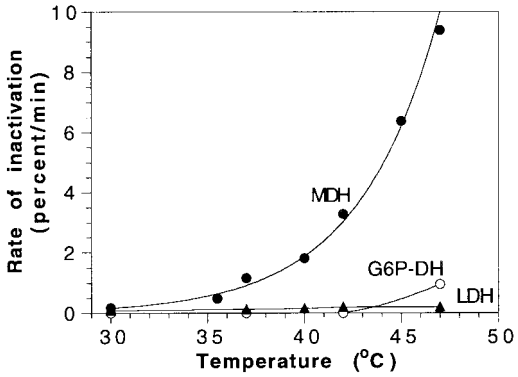

FIGURE 2: Effect of temperature on the stability of native enzymes. Relative rates of enzymatic inactivation at increasing temperatures: G6P-DH (○), MDH (●), and LDH (▲).

enzymes were specifically reactivated by the KJE chaperones (Figure 1, insets), compared to very low levels of spontaneous refolding without chaperones (not shown). The KJE-mediated refolding activity strongly depended on the temperature and on the type of protein substrate. Between 30 and 40 °C, the refolding rates of G6P-DH, or of MDH, strongly decreased and the reaction reached complete arrest at 40 °C and above (Figure 1a,b). Whereas the rate of KJE-mediated refolding of LDH first increased between 30 and 37 °C, it also strongly decreased (9-fold) between 37 and 47 °C (Figure 1c).

The rates of thermal inactivation of native G6P-DH, MDH, and LDH were measured under the same conditions (Figure 2). Noticeably, the enzymatic activity of native G6P-DH and LDH was virtually unaffected by the elevated temperatures that strongly affected KJE-mediated refolding of denatured G6P-DH and LDH (Figure 1a,c). Whereas KJE-mediated refolding of MDH was completely inhibited at 40 °C, the stability of native MDH was only mildly affected by the same temperature (Figure 2).

The Nature of the Substrate Protein Controls Folding Arrest during Heat Shock. The fate of the folding substrates during inhibition of the chaperone activity at high temperatures was addressed by examining rates and yields of recovered enzymatic activity, after various increasing incubation periods with ATP at 47 °C. Remarkably, equal amounts of G6P-DH were efficiently recovered after 30 min of heat shock, during which the KJE-mediated protein-folding activity was completely inhibited (Figure 3a), as compared to that recovered without heat shock. Hence, G6P-DH, during heat shock, appears to have been kept in a competent state, which was efficiently refolded after the stress. Lower yields of recovered MDH and LDH were obtained when the incubation time at 47 °C was increased (Figure 3b,c), suggesting that KJE chaperones are less efficient at keeping MDH and LDH than at keeping G6P-DH in a refoldable state during stress (Figure 3a). Thus, the efficiency of protein-folding arrest by KJE chaperones at high temperatures depends on the nature of the protein substrate.

Interactions among DnaK, DnaJ, and GrpE Are Thermosensitive. The ability of DnaJ and/or GrpE to separately and synergistically activate the ATPase activity of DnaK (*16*) was exploited to estimate the degree of interaction among DnaK, DnaJ, and GrpE at various temperatures. Limiting concentrations of DnaJ activated the ATPase activity of

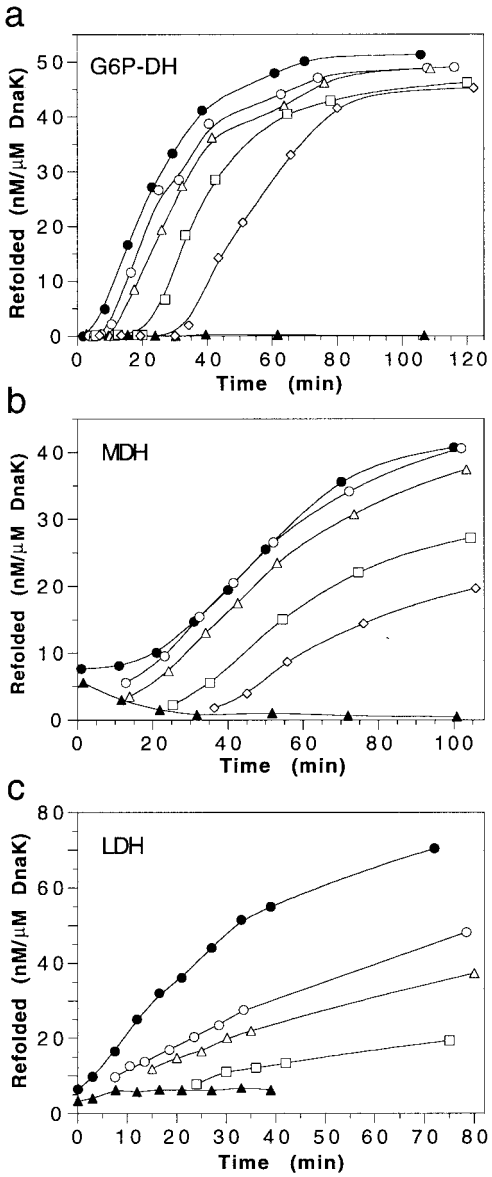

FIGURE 3: Recovery of enzymatic activity after inhibition of KJE-mediated refolding at high temperature. (a) Urea- and heat-denatured G6P-DH, (b) heat-denatured MDH, and (c) urea-denatured LDH first interacted with DnaK, DnaJ, and GrpE as in Figure 1, and were incubated with ATP at 47 °C (▲) for 0 (●), 5 (○), 10 (△), 20 (□), and 30 min (◇) and then cooled to 30 °C. The enzymatic activity was measured during and after the various heat-shock periods.

DnaK−GrpE at all temperatures (Figure 4a), but the DnaJ activation, which was 17-fold at 30 °C, decreased to 6-fold at 47 °C (Figure 4a, inset). Similarly, limiting concentrations of GrpE activated the ATPase activity of DnaK−DnaJ at all temperatures (Figure 4b), but the GrpE activation which was 8-fold at 30 °C decreased to 5-fold at 47 °C (Figure 4b, inset). The constants (K_{Aapp}) for activation of DnaK−GrpE by DnaJ, and of DnaK−DnaJ by GrpE, were derived at various temperatures from DnaJ and GrpE dose−response curves of the DnaK ATPase activity, measured in the presence of excess amounts of the other cochaperone and a saturative concentration of ATP (Figure 5a,b). Both apparent affinities ($1/K_{Aapp}$) of GrpE for DnaK−DnaJ and of DnaJ for DnaK−GrpE were 3−4 times lower at 47 °C than at 30 °C (Figure 5c).

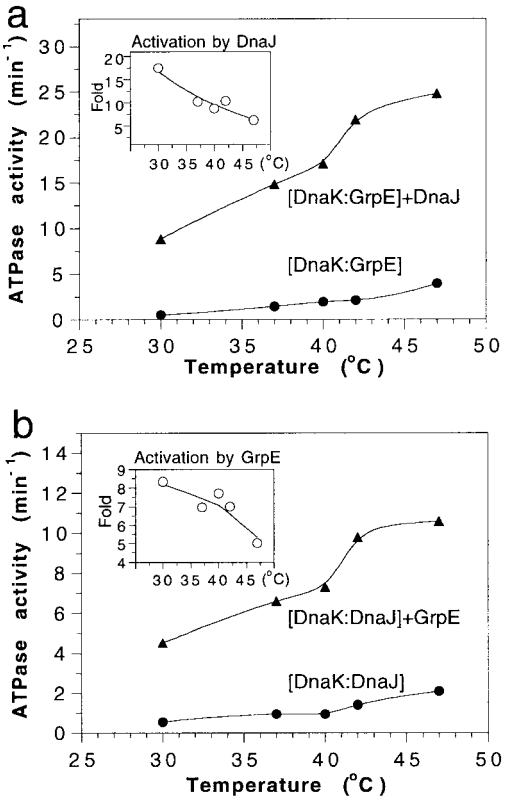

FIGURE 4: Effect of temperature on the DnaK ATPase activity. (a) Temperature-dependent rate of ATP hydrolysis by DnaK (3.5 μM) in the presence of GrpE (1.75 μM), without (●) or with (▲) DnaJ (0.875 μM). (a, inset) Temperature-dependent activation of DnaK−GrpE ATPase by DnaJ. (b) Temperature-dependent rate of ATP hydrolysis by DnaK (3.5 μM) in the presence of DnaJ (3.5 μM) without (●) or with (▲) GrpE (0.35 μM). (b, inset) Temperature-dependent activation of DnaK−DnaJ ATPase by GrpE.

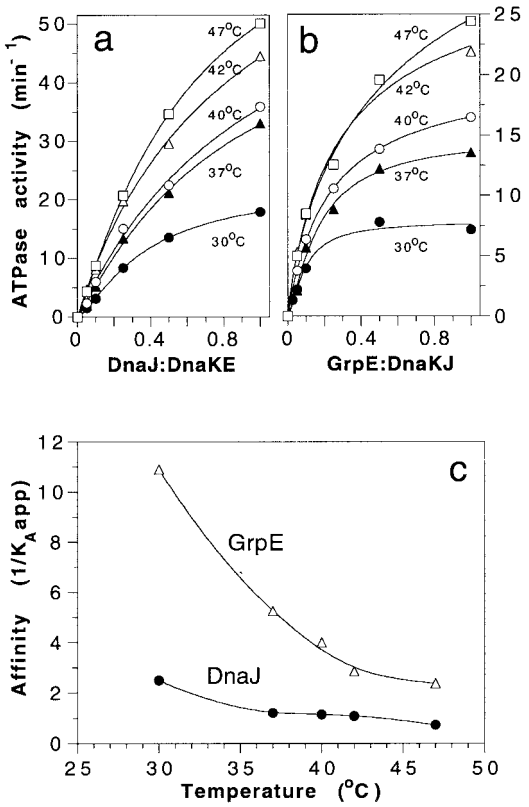

FIGURE 5: Effect of temperature on the apparent affinity of GrpE and DnaJ for DnaK. (a) Effect of increasing concentrations of DnaJ on the rates of ATP hydrolysis of DnaK (3.5 μM) in the presence of GrpE (1.75 μM) at increasing temperatures. (b) Effect of increasing concentrations of GrpE on the rates of ATP hydrolysis of DnaK (3.5 μM) in the presence of DnaJ (3.5 μM) at increasing temperatures. (c) Apparent affinity constants ($1/K_{Aapp}$) of DnaJ for DnaK−GrpE (●) and of GrpE for DnaK−DnaJ (▲) at increasing temperatures. K_{Aapp} values are the effective concentrations of DnaJ, or GrpE, which caused half of the maximal activation of the DnaK−GrpE ATPase, or DnaK−DnaJ ATPase, calculated from the results of panels a and b.

Excess Cochaperone Does Not Restore Protein Folding at High Temperatures. We next addressed the possible correlation between protein-folding arrest and the decreased apparent affinities of GrpE and DnaJ for DnaK at high temperatures. When KJE-mediated refolding of thermostable LDH was measured in the presence of a nonphysiological excess concentration of DnaJ ($8K_{Aapp}$), or of GrpE ($10K_{Aapp}$), LDH refolding rates were inhibited 4- and 6-fold, respectively (Figure 6a), compared to a K:J:E ratio of 5:1:0.5 (Figure 6a, hatched line as in Figure 1c). Interestingly, when the temperature was increased, the protein-folding inhibition by excess cochaperones was not relieved, as expected from the compensation of the decreased affinity of the cochaperone for DnaK, but became even more inhibited.

Excess GrpE Affects the Stability of the Chaperone−Protein Complex during Stress. In the presence of a nonphysiological concentration of GrpE ($6.7K_{Aapp}$), KJ-bound G6P-DH became much more sensitive to heat shock at 47 °C (Figure 6b) than in the presence of limiting GrpE ($0.7K_{Aapp}$) (Figure 6b, hatched line as in Figure 3a). Unlike limiting GrpE, high GrpE strongly lowered the rates and yields of G6P-DH recovery after increasing periods of stress. This indicates that at all temperatures, GrpE can modulate the strength of the interaction between folding substrates and the chaperone.

Protein-Folding Activity at High Temperatures Is Regulated by GrpE. The refolding activity of KJE was measured at 30 °C in the presence of increasing concentrations of GrpE and compared to the refolding activity at near, but not completely, inhibitory high temperatures (Figure 7). Low GrpE concentrations first activated and then, after an optimum near physiological concentration, high GrpE concentrations inhibited the rate of protein folding. When the GrpE concentration was 10−20 times lower than that of DnaK as in the cell, GrpE activations of both ATPase and protein-folding activities were in good correlation with one another. However, when the GrpE concentrations exceeded the physiological range, protein-folding activity decreased, while the ATPase activity further increased (Figure 5b).

Modulating the GrpE concentration further revealed the sensitivity of the chaperone system to the nature of the unfolded substrates at various temperatures. In the presence of low activatory or high inhibitory concentrations of GrpE, rates of G6P-DH and MDH refolding were consistently lower at high temperatures, as compared to those at 30 °C (Figure 7a,b). In contrast, high temperatures (44 °C) potentiated both the activation by limiting GrpE and the inhibition by excess GrpE of LDH refolding (Figure 7c). Hence, in the case of thermostable LDH, the optimal refolding activity was 2 times faster at 44 °C than at 30 °C, and excess GrpE was much more inhibitory than in the case of G6P-DH and MDH. The GrpE concentration is therefore central to the temperature-

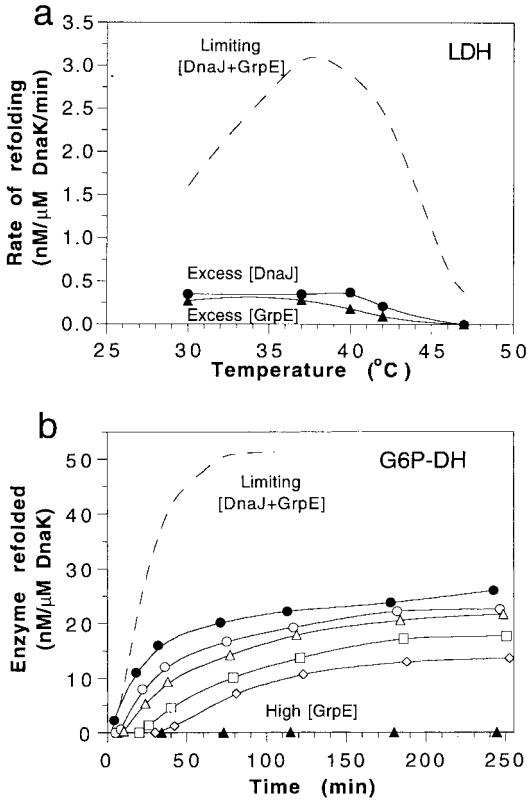

FIGURE 6: Effect of temperature on chaperone-mediated refolding in the presence of excess cochaperones. (a) Rates of KJE-mediated refolding at different temperatures of urea-denatured LDH in the presence of DnaK (3.5 μM), excess DnaJ (10.5 μM), and limiting GrpE (●) or limiting DnaJ (0.7 μM) and excess GrpE (5.25 μM) (▲). The hatched line represents the KJE-mediated refolding in the presence of limiting amounts of DnaJ (0.7 μM) and GrpE (0.35 μM) as a control from Figure 1c. After equilibration at the indicated temperatures, protein refolding was initiated by addition of ATP and PK. (b) Time-dependent refolding during and after inhibition at 47 °C of KJE-bound G6P-DH as in Figure 3a, but in the presence of a high concentration of GrpE (3.5 μM). The hatched line represents the time-dependent refolding at 30 °C in the presence of limiting DnaJ and GrpE as in Figure 3a.

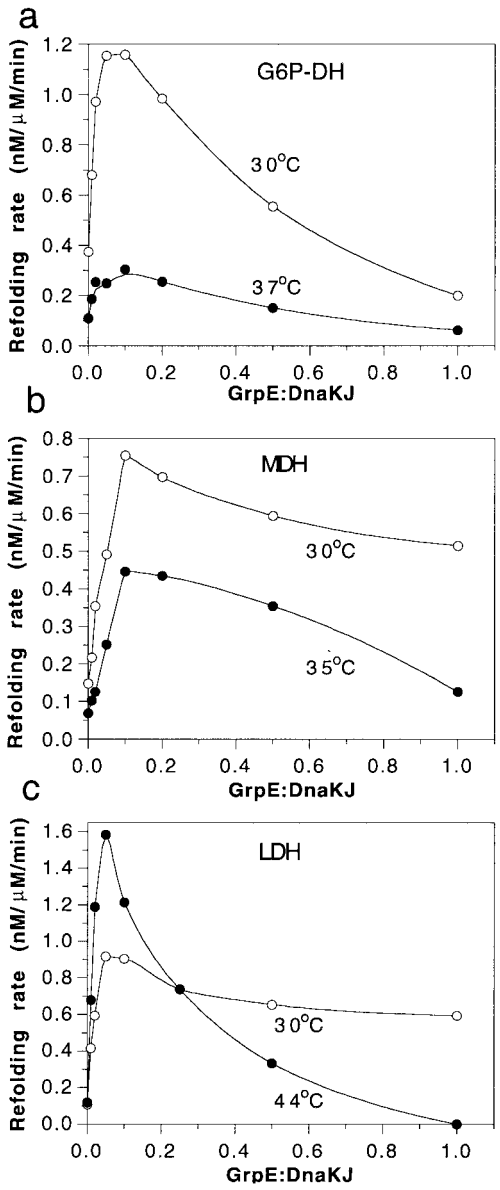

FIGURE 7: Effect of GrpE concentration on the rate of KJE-mediated protein refolding. Rates of KJE-mediated refolding of (a) urea- and heat-denatured G6P-DH, (b) heat-denatured MDH, and (c) urea-denatured LDH as in Figure 1, in the presence of DnaK (3.5 μM), DnaJ (0.7 μM), and increasing concentrations of GrpE at low temperatures (30 °C) and at elevated temperatures (37, 35, and 44 °C, as indicated).

dependent mechanism of selective protein-folding arrest on KJE chaperones.

DISCUSSION

The correct folding of proteins is thought to result from the kinetic competition between partially folded intermediates undergoing productive on-pathway refolding, leading to biologically active native proteins, and abortive off-pathway misfolding, leading to protein aggregation (*30*, *31*). During heat shock, thermosensitive proteins are subject to unfolding and heat-shock chaperones are concomitantly synthesized in large amounts (*21*). Heat-induced exposure of hydrophobic residues from the core of heat-labile proteins may lead to protein aggregation, unless the hydrophobic residues are allowed to interact with a chaperone surface (*17*, *32*−*36*).

We examined here the protein-folding activity of KJE chaperones from *E. coli* under simulated heat-shock and physiological temperatures. The rate of KJE-mediated refolding of denatured proteins was found to gradually decrease as the temperature was increased. Since the stability of the native substrates was less sensitive to elevated temperature than the chaperone-mediated protein-refolding activity, inhibition was unlikely to result from the denaturation of reactivated native substrates. The degree of protein-folding inhibition during heat shock was more pronounced in the case of G6P-DH and MDH than in the case of LDH, suggesting that the nature of the interaction between unfolded proteins and KJE chaperones controls the mechanism of KJE-mediated protein binding, folding, and release, in particular during stress. Remarkably, the same amount of G6P-DH was recovered after extensive incubations at 47 °C with KJE and ATP as was recovered without heat-shock. This demonstrates that the inhibition of KJE-mediated protein folding at high temperatures is a reversible process and confirms previous observations (*37*, *38*) that KJE are thermostable proteins that remain unaffected by the heat-shock conditions used in this study. Hence, a denatured protein can become arrested in a folding-competent state during stress and resume KJE-mediated refolding after the

stress. In addition, we observed that, when the temperature was increased, the rate of ATP hydrolysis by DnaK also increased, while the rate of KJE-mediated protein folding decreased. This implies that during heat shock the ATPase activity becomes gradually uncoupled from the protein-folding activity. The degree of coupling and/or uncoupling depended on the nature of the bound protein; at 47 °C, uncoupling was partial in the case of LDH and complete in the case of MDH and G6P-DH refolding.

A similar behavior of reversible protein-folding arrest during heat shock has been demonstrated in the case of human cytosolic HSP70/hdj-1 chaperones (*39*). Denatured proteins can also become reversibly arrested during heat shock on the GroEL chaperonin (*27*, *40*), by a mechanism of reversible changes in the affinity of GroES for GroEL (*27*). We addressed here the possibility that protein-folding arrest on KJE is also caused by changes in the affinity of cochaperones GrpE and DnaJ for DnaK. However, unlike in the case of GroELS chaperonins, KJE-mediated refolding of LDH was not restored at high temperatures by an excess of cochaperones, suggesting that decreased affinity of GrpE and DnaJ for DnaK is not the primary mechanism for the observed reversible protein-folding arrest during heat shock. It is possible, however, that when both cochaperones are limiting, like in the cell, their decreased affinity for DnaK can reduce the level of futile ATP hydrolysis during stress.

Analysis of protein-folding rates in the presence of increasing GrpE concentrations revealed a biphasic pattern for all proteins tested at all temperatures. Protein folding and ATPase were coactivated by low GrpE concentrations. Concentrations of GrpE above the physiological range inhibited protein folding but not the ATPase activity. This corroborates an observation by Packschies et al. (*41*), who found that excess GrpE can reduce the yields of KJE-refolded luciferase. Depending on the nature of the bound protein, but independent of the temperature, optimal protein-folding activity was observed at GrpE:DnaK ratios of 0.1−0.2, close to the reported cellular ratios (*12*). Since GrpE binds DnaK as a dimer (*19*, *20*, *25*), this implies that in the cell at equilibrium less than 10% of the DnaK molecules can be associated with GrpE at any given time. Thus, optimal KJE-mediated protein folding in the cell may involve highly dynamic exchanges of GrpE dimers with protein−KJ complexes.

When the GrpE concentration was limiting, like in the cell, the ability of the KJE system to fold non-native proteins, or to arrest bound proteins in a refoldable state at high temperatures, primarily depended on the interplay between the temperature and the nature of the folding substrate. In the case of LDH, which apparently formed a weak complex with KJ, high temperatures and GrpE jointly destabilized the chaperone−LDH complex, resulting in the potentiation of folding activation by low GrpE concentrations and of folding inhibition by high GrpE concentrations. In the case of G6P-DH, which apparently formed a strong complex with KJ, high temperatures may further stabilize the association and partially counteracted the destabilization effect of high GrpE concentrations.

Palleros et al. (*42*) have shown that binding between DnaK and a permanently unfolded form of α-lactalbumin does not withstand a temperature increase from 40 to 45 °C. On the other hand, excess GrpE promotes the destabilization of DnaK−λP and DnaK−α-lactalbumin complexes, even in the absence of nucleotides (*19*, *20*). In addition to the major interaction of the GrpE dimer with the ATPase domain of DnaK, a second type of direct interaction has also been suggested between GrpE and the protein-binding site of DnaK (*20*). However, the destabilization effect by GrpE dimers was counterbalanced by the presence of DnaJ (*19*). Our results are compatible with such an effect of GrpE on the stability of the KJ−protein complex. At low concentrations, GrpE expectedly enhanced the nucleotide exchange and promoted the release of substrate species capable of reactivation. In contrast, high GrpE concentrations seemed to promote the conversion of protein substrates into kinetically trapped species that are unable to reactivate.

Whereas whole proteins bind and become sequestered under the cap of $GroES_7$ within the cage-like structure of $GroEL_{14}$ (*43*), DnaK and DnaJ chaperones can bind denatured proteins at locally extended motives of exposed residues on a single polypeptide (*13*, *44*). Hence, when present in large excess, several DnaK and DnaJ molecules may bind a single molecule of protein substrate, at several high-affinity binding sites that randomly exist in all unfolded proteins of the size of MDH, LDH, and G6P-DH (*44*). This implies that, during the KJE-mediated refolding, single DnaK or KJ complexes may dissociate from the protein substrate independent of one another, while assisting the gradual refolding of different regions in the polypeptide. At the same time, other KJ complexes could maintain the whole polypeptide in a nonaggregated state. This mechanism is very different from that of $GroEL_{14}$ chaperonin, which requires repeated cycles of complete release and rebinding of the substrate polypeptide, within or outside the chaperone cavity (*30*).

The dissociation of KJE at high temperatures from a thermostable protein such as LDH may not necessarily be deleterious to the cell, since many such released proteins may yet be reactivated into life-sustaining stable enzymes during stress and, by doing so, liberate the KJE chaperone to bind aggregation-prone proteins. In contrast, thermolabile proteins that form strong associations with KJE chaperones may become even more strongly bound at higher temperatures, possibly following the unraveling of additional hydrophobic high-affinity sites.

There is increasing in vitro and in vivo evidence that the various chaperone systems in the cell collaborate in the folding of nascent proteins and in the refolding of stress-denatured proteins. Depending on their intrinsic nature, denatured proteins can be bound, distributed, and moved differently between the various components of a multichaperone network of GroEL/GroES, KJE, small HSPs, Hsp90, etc. (*14*, *36*, *39*, *45*−*48*). GroEL/GroES proteins are thermoregulated heat-shock chaperones which can arrest the folding and release of proteins during heat shock and resume protein-folding activity after stress (*27*, *40*). Our results, showing selective reversible protein-folding arrest on KJE chaperones, suggest that this biologically sound behavior can now be extended to KJE chaperones, which often cooperate with GroEL/GroES in the chaperone network (*45*, *47*). Thus, chaperones may collaborate not only in the sequential folding of proteins but also in the selective binding and stabilization during stress of various protein intermediates in a folding-

competent state, and in their optimal reactivation after heat stress.

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