Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 10345–10349, October 1994
Biochemistry

The ATP hydrolysis-dependent reaction cycle of the *Escherichia coli* Hsp70 system—DnaK, DnaJ, and GrpE

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Communicated by James E. Rothman, July 18, 1994 (received for review June 27, 1994)

ABSTRACT **Molecular chaperones of the Hsp70 class bind unfolded polypeptide chains and are thought to be involved in the cellular folding pathway of many proteins. DnaK, the Hsp70 protein of *Escherichia coli*, is regulated by the chaperone protein DnaJ and the cofactor GrpE. To gain a biologically relevant understanding of the mechanism of Hsp70 action, we have analyzed a model reaction in which DnaK, DnaJ, and GrpE mediate the folding of denatured firefly luciferase. The binding and release of substrate protein for folding involves the following ATP hydrolysis-dependent cycle: (*i*) unfolded luciferase binds initially to DnaJ; (*ii*) upon interaction with luciferase–DnaJ, DnaK hydrolyzes its bound ATP, resulting in the formation of a stable luciferase–DnaK–DnaJ complex; (*iii*) GrpE releases ADP from DnaK; and (*iv*) ATP binding to DnaK triggers the release of substrate protein, thus completing the reaction cycle. A single cycle of binding and release leads to folding of only a fraction of luciferase molecules. Several rounds of ATP-dependent interaction with DnaK and DnaJ are required for fully efficient folding.**

Protein folding in the cell is dependent on the ability of molecular chaperones to prevent unproductive interactions between folding polypeptides (1–3). Two main classes of molecular chaperones, the members of the heat shock protein 70 (Hsp70) and heat shock protein 60 (Hsp60) families have been implicated in protein folding in various cell types and organelles. Hsp70 and Hsp60 are able to cooperate in a sequential pathway of assisted polypeptide chain folding in mitochondria and presumably also in the *Escherichia coli* cytosol (3). The Hsp70s recognize short extended peptide sequences enriched in hydrophobic amino acid residues (4–6). They are thought to stabilize the unfolded state of proteins during translation and membrane translocation and to release their bound substrate upon ATP binding and hydrolysis (for review, see refs. 1–3). The ATPase activity of the Hsp70 homolog of *E. coli*, DnaK, is known to be regulated by the chaperone protein DnaJ and the cofactor GrpE (7, 8). However, most work on the binding of Hsp70s to substrates has been carried out with purified Hsp70 protein alone (9–12). These studies have also been performed using peptides or chemically modified protein substrates that are unable to fold. Recent results indicate that DnaJ and GrpE homologs are required for many, if not all, biological functions of Hsp70s (2, 13–16), making it important to understand the interaction of substrates with DnaK in the presence of these regulators. We have therefore analyzed the role of DnaK as a chaperone in protein folding in context with DnaJ and GrpE by using a model reaction in which all three proteins are required to mediate the refolding of denatured firefly luciferase *in vitro*. Our results establish the nucleotide-dependent reaction cycle of the complete Hsp70 system.

METHODS

Proteins. DnaK (17), DnaJ (18), and GrpE (19) proteins were purified as described. The fragment consisting of DnaJ residues 1–127 was expressed in *E. coli* from a recombinant gene encoding the truncated protein and purified by the protocol used to purify DnaJ. Protein concentrations were determined by quantitative amino acid analysis. Firefly luciferase (≈90% pure), α-casein, and reduced carboxymethylated α-lactalbumin were from Sigma.

Refolding Experiments. Firefly luciferase was denatured at 25 μM in buffer A (6 M guanidinium hydrochloride/30 mM Tris·HCl, pH 7.4/5 mM dithiothreitol by incubation for 30 min at room temperature. Unfolded luciferase was diluted 1:100 to 0.25 μM (final concentration) into buffer B (10 mM Mops, pH 7.2/50 mM KCl) at 25°C containing Mg^{2+}ATP (5 mM $MgCl_2$/1 mM ATP) or Mg^{2+}-adenosine 5′-[β,γ-imido] triphosphate (p[NH]ppA) (5 mM $MgCl_2$/1 mM p[NH]ppA), 0.25 μM DnaJ, and 1.25 μM DnaK as indicated. Refolding was initiated by addition of GrpE to 1.25 μM. Luciferase activity was measured using the Promega luciferase assay system and a Bio-Orbit luminometer (20).

In Fig. 3, refolding was measured from a reisolated luciferase–DnaK–DnaJ complex formed in the presence of MgATP as described above, then separated from free nucleotide by a wash cycle consisting of three steps of a 1:20 dilution with buffer B, and concentration using a Centricon-30 filter device (Amicon). The original protein concentration was restored, and refolding was measured 1 hr after addition of nucleotides and GrpE, as indicated. To determine the percentage of luciferase aggregating, aliquots of each reaction mixture were taken 15 min after addition of nucleotides and GrpE and centrifuged (10 min at 30,000 × *g*), and the amount of luciferase in the pellet and supernatant fractions was quantified by SDS/PAGE and laser densitometry. Sedimentation of DnaJ and DnaK was negligible and was not affected by the addition of nucleotides or GrpE. In experiments containing competitive inhibitors for binding to DnaK or DnaJ, the luciferase–DnaK–DnaJ complex was initially formed as described above and separated from free nucleotide. After addition of the indicated concentrations of reduced carboxymethylated α-lactalbumin, α-casein, or DnaJ residues 1–127 to the reisolated complex, refolding was initiated by addition of GrpE and nucleotide. Refolding was measured after 1 hr.

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Abbreviation: p[NH]ppA, adenosine 5′-[β,γ-imido]triphosphate.

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Column Chromatography. Denatured luciferase was diluted to 1 μM into buffer B containing 1 μM DnaJ. After a 5-min incubation at 25°C, DnaK (1 μM) alone or DnaK and MgATP (5 mM $MgCl_2$/1 mM ATP) were added. After a further 10-min incubation, 10% of the sample was removed for determination of protein recoveries, and the remainder was loaded onto a 1 × 30 cm Sephacryl S-300 column (Pharmacia) equilibrated in 10 mM Mops, pH 7.2/250 mM KCl/0.008% Tween 20. When present in the initial reaction, MgATP was also added to the column buffer. Fractions (0.5 ml) were collected, and proteins were precipitated by 10% (wt/vol) trichloroacetic acid and analyzed by SDS/PAGE followed by Coomassie blue staining and laser densitometry.

Thin Layer Chromatography. Reactions were prepared in buffer B containing DnaK alone or DnaK/DnaJ and denatured luciferase. Concentrations of proteins were 5 μM DnaK, 1 μM DnaJ, and 1 μM luciferase, and 5 mM $MgCl_2$ and 100 μM [α-^{32}P]ATP (20 mCi/mmol, final specific activity; 1 Ci = 37 GBq) were added. After a 10-min incubation at 25°C during which ≈10 μM ADP was generated, 5% of each reaction mixture was removed for determination of protein concentration (21). Free nucleotide was separated from DnaK-bound nucleotide as described above by a wash cycle using a Centricon-30 filter. After the third centrifugation step, the initial protein concentration was restored. The sample containing the luciferase–DnaK–DnaJ complex was split into four reaction mixtures, to which GrpE was added to 0, 1, 5, and 15 μM, respectively. After a second incubation of 5 min at 25°C, bound nucleotide was again separated from free nucleotide by a second wash cycle, the original protein concentration was restored and 2-μl samples were spotted onto a Polygram CEL 300 thin layer chromatography plate. Migration was performed using a buffer system consisting of 0.5 M formic acid and 0.5 M lithium chloride (22), and the plate was subjected to autoradiography.

RESULTS

The 62-kDa protein firefly luciferase was completely unfolded in 6 M guanidinium hydrochloride and then diluted into a buffer containing DnaK, DnaJ, GrpE, and MgATP. This resulted in highly efficient refolding of luciferase to the active enzyme (Fig. 1), whereas in the absence of chaperones, most of the protein did not reach the native state. To understand the mechanism of this reaction, its essential components and the order in which they are required was determined. The chaperone-mediated refolding was strictly dependent on MgATP and was not supported by the nonhydrolyzable analog of ATP, p[NH]ppA (AMP-PNP). Maximum reactivation was obtained at a molar ratio of luciferase/DnaK/DnaJ of 1:5:1, close to the ratio of the cellular concentrations of DnaK and DnaJ (3). Once diluted into buffer containing DnaK, DnaJ, and MgATP, unfolded luciferase could be maintained in a folding-competent conformation for >1 hr until initiation of refolding by the addition of GrpE (data not shown). Significantly, DnaK was not required at the time of dilution of luciferase from denaturant but could be added subsequently (Fig. 1). On the other hand, luciferase aggregated (as measured by the sedimentation of the protein upon centrifugation; data not shown) and could not be refolded when DnaK alone was present, indicating that DnaJ must interact first with the unfolded polypeptide to prevent its aggregation. This result and the recent demonstration that DnaJ can be crosslinked to short ribosome-bound polypeptides (23) suggest that in the cell, DnaJ may be the first molecular chaperone to bind nascent chains during synthesis.

Gel-exclusion chromatography on Sephacryl S-300 revealed that prior to the initiation of refolding by GrpE, denatured luciferase was stabilized in a complex with DnaK and DnaJ. Surprisingly, formation of this complex was dependent on MgATP, although previous analyses of polypeptide binding to Hsp70s in the absence of DnaJ had shown that MgATP dissociates the complex between the chaperone and its substrate (11, 12). In the absence of MgATP, luciferase and DnaJ coeluted from the size-exclusion column without affecting the mobility of DnaK (Fig. 2*A*). The luciferase–DnaJ complex fractionated over a broad molecular mass range and was eluted later from the column than expected, suggesting that the unfolded luciferase is incompletely shielded by DnaJ alone and is retained by interaction with the column matrix. Only in the presence of hydrolyzable ATP did a large fraction of luciferase, DnaK, and DnaJ coelute in a higher molecular mass complex of ≈250 kDa (Fig. 2*B*). The luciferase in this complex was unfolded, as it was enzymatically inactive and highly sensitive to proteinase K (data not shown).

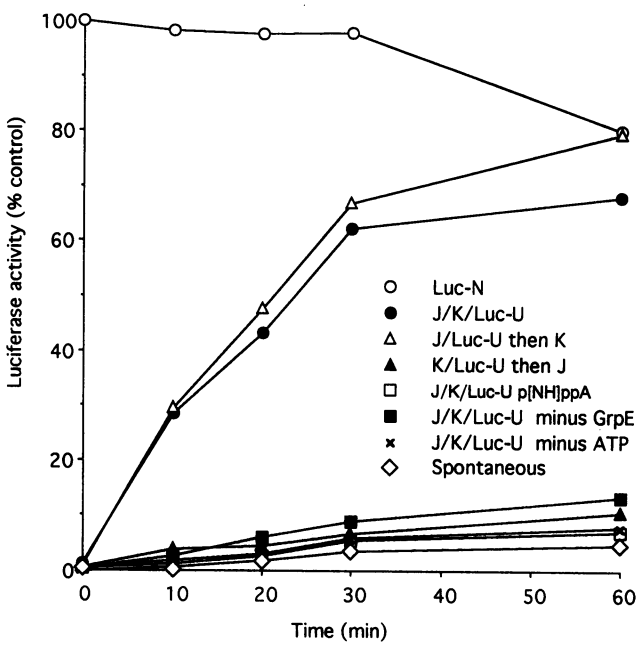

FIG. 1. Reactivation of denatured firefly luciferase (Luc-U) by DnaK, DnaJ, and GrpE. Activity was measured after dilution of luciferase from denaturant into solution containing DnaJ (J) and DnaK (K) in the absence (×) or presence (●) of MgATP, DnaJ/MgATP only with DnaK added after 5 min (△), DnaK/MgATP only with DnaJ added after 5 min (▲), or DnaJ/DnaK with p[NH]ppA added in place of ATP (□). Refolding was initiated by addition of GrpE 10 min after luciferase dilution. GrpE was omitted from one reaction mixture containing DnaK, DnaJ, and MgATP (■). The activity of native luciferase over the course of the experiment (Luc-N, ○) and the spontaneous refolding of luciferase at a 50-fold higher dilution (◇) are indicated. Spontaneous refolding of luciferase at the dilution used when chaperones were present resulted in regaining <5% activity after 1 hr and was unaffected by the presence of MgATP.

To analyze the nucleotide state of DnaK when bound to DnaJ and unfolded luciferase, this ternary complex was prepared in the presence of [α-^{32}P]ATP and separated from free nucleotide by microfiltration. Analysis by thin layer chromatography indicated that ADP was the sole nucleotide bound by DnaK (Fig. 3*A*, lane 4). In contrast, DnaK in the absence of DnaJ and denatured luciferase was found to have ADP and ATP bound in roughly equal amounts (lane 2), indicating that in the absence of DnaJ and folding substrate, the hydrolysis and nucleotide release steps occur at approximately the same rate. The effect of GrpE on DnaK has previously been investigated in the absence of substrate protein. Under these conditions, GrpE stimulates ADP–ATP exchange in DnaK (7). We therefore reasoned that the requirement for GrpE in the refolding reaction may be to

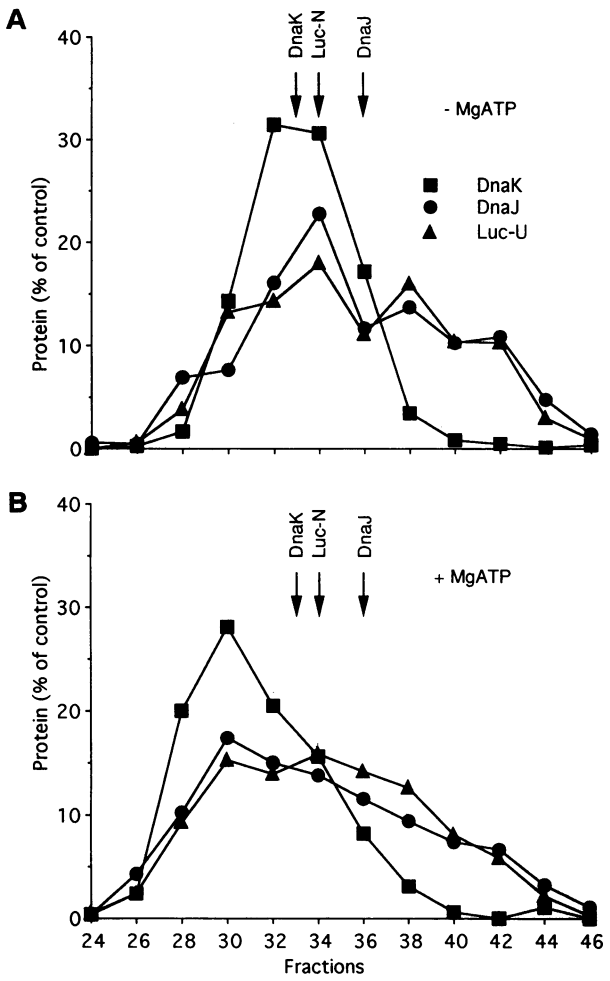

FIG. 2. Formation of a ternary luciferase–DnaK–DnaJ complex in the presence of hydrolyzable ATP. Size-exclusion chromatography of luciferase after dilution of the denatured protein into DnaK- and DnaJ-containing solution in the absence (*A*) or presence (*B*) of MgATP. Luciferase (▲), DnaJ (●), and DnaK (■) quantities are expressed as percent of the total protein eluted from the column (≈90% of the applied protein). The positions of the peak fractions of native luciferase (LucN), DnaK, and DnaJ applied together on the same column are indicated. Note that chromatography was performed using a 1:1:1 molar ratio of luciferase/DnaK/DnaJ, which is suboptimal for folding, to observe the shift in the migration of DnaK. The stoichiometry of the components in the 250-kDa complex has not yet been determined.

release bound nucleotide to permit substrate protein release. Addition of GrpE indeed triggered the dissociation of bound ADP from DnaK in a concentration-dependent manner (lanes 4–7).

The approach of separating the luciferase–DnaK–DnaJ complex from free nucleotide was used to dissect the nucleotide requirement for the release of luciferase from DnaK and DnaJ. When GrpE alone was added to the isolated complex under the same conditions that triggered ADP release from DnaK, no reactivation of luciferase above background levels occurred (Fig. 3*B*). Luciferase could be refolded, however, when both MgATP and GrpE were added. We tested whether the release of luciferase from DnaK–DnaJ involved ATP hydrolysis. Interestingly, the addition of the nonhydrolyzable ATP analog p[NH]ppA resulted in an intermediate level of reactivation, reaching 35% of the activity obtained in the presence of ATP. This was not due to a slow or incomplete release of luciferase. Another 30% of the luciferase that did not fold upon addition of GrpE and p[NH]ppA was in fact released from the chaperone-bound state but aggregated (Fig. 3*B*). Upon centrifugation, it was recovered in the pellet (Fig. 3*B*) while DnaK, DnaJ, and GrpE remained soluble (data not shown). The likely interpretation of this result is that the addition of p[NH]ppA and GrpE allowed the release of luciferase from DnaK and DnaJ but not the reformation of the ternary complex. That only a fraction of the luciferase folded upon a single release event suggests that several cycles of release and rebinding are necessary for the efficient folding of luciferase.

If folding does indeed require multiple cycles of dissociation and reformation of the substrate protein–DnaK–DnaJ complex, it should be possible to inhibit the reaction with polypeptide substrates that act as competitive inhibitors for the binding of unfolded luciferase to either DnaK or DnaJ. It has previously been shown that reduced carboxy-methylated α-lactalbumin, a permanently unfolded form of α-lactalbumin, binds specifically to DnaK (10, 24) but does not bind DnaJ (24). In contrast, α-casein, a protein that exposes a significant amount of hydrophobic surface, binds DnaJ but not DnaK (24). As shown in Fig. 3*C*, each of these reagents, when added to the isolated ternary complex, inhibited refolding upon addition of MgATP and GrpE in a concentration-dependent manner. However, neither reagent inhibited the reduced amount of refolding that occurred, apparently upon a single release event, triggered by the addition of GrpE and p[NH]ppA. In addition, we tested the effect on luciferase refolding of an N-terminal fragment of DnaJ (residues 1–127) that stimulates the ATPase activity of DnaK and competitively inhibits the binding of DnaJ to DnaK (A.S. and J.F., unpublished data). This DnaJ fragment also partially inhibited the reactivation of luciferase upon addition of MgATP but had no effect on the smaller amount of reactivation obtained upon addition of p[NH]ppA. These results support the conclusion that luciferase must undergo several ATP-dependent cycles of binding to DnaJ and DnaK for efficient folding.

DISCUSSION

Our results are summarized in a model of the DnaK reaction cycle (Fig. 4). When not in a complex with DnaJ and substrate protein, a large fraction of DnaK is in the ATP state with low substrate affinity (10). Unfolded protein initially binds to DnaJ (step 1). Substrate-bound DnaJ then interacts with DnaK. This activates the ATP hydrolytic activity of DnaK (step 2) and stabilizes its high-affinity ADP state (10). As a result, a substrate protein–DnaK–DnaJ complex is formed in which DnaJ is likely to maintain its direct interaction with the bound protein. DnaK remains associated until GrpE causes ADP dissociation (step 3). The GrpE-induced dissociation of ADP alone may labilize the interaction of substrate protein, DnaK, and DnaJ. This is suggested by the observation that GrpE alone is sufficient to trigger the transfer of another unfolded protein, bovine rhodanese, from DnaK–DnaJ to the chaperonin GroEL (T.L., unpublished data). For luciferase, the binding of ATP (or p[NH]ppA) to DnaK releases the substrate protein, resulting in folding to the native state of a fraction of the released protein molecules (step 4). At this step, the chaperones do not function solely by reducing the concentration of unfolded protein, thereby making aggregation less favorable. We can conclude this from the observation that denatured luciferase does not refold spontaneously, even at 25- to 50-fold higher dilutions (Fig. 1). A fraction of luciferase molecules may undergo a partial folding reaction either in association with DnaK and DnaJ or during protein release. Folding after a single cycle of release is inefficient, however, as a significant amount of the dissociated protein aggregated. ATP hydrolysis by DnaK leads to the reformation of the stable ternary complex, thus, preventing the aggregation of those protein molecules that are unable to

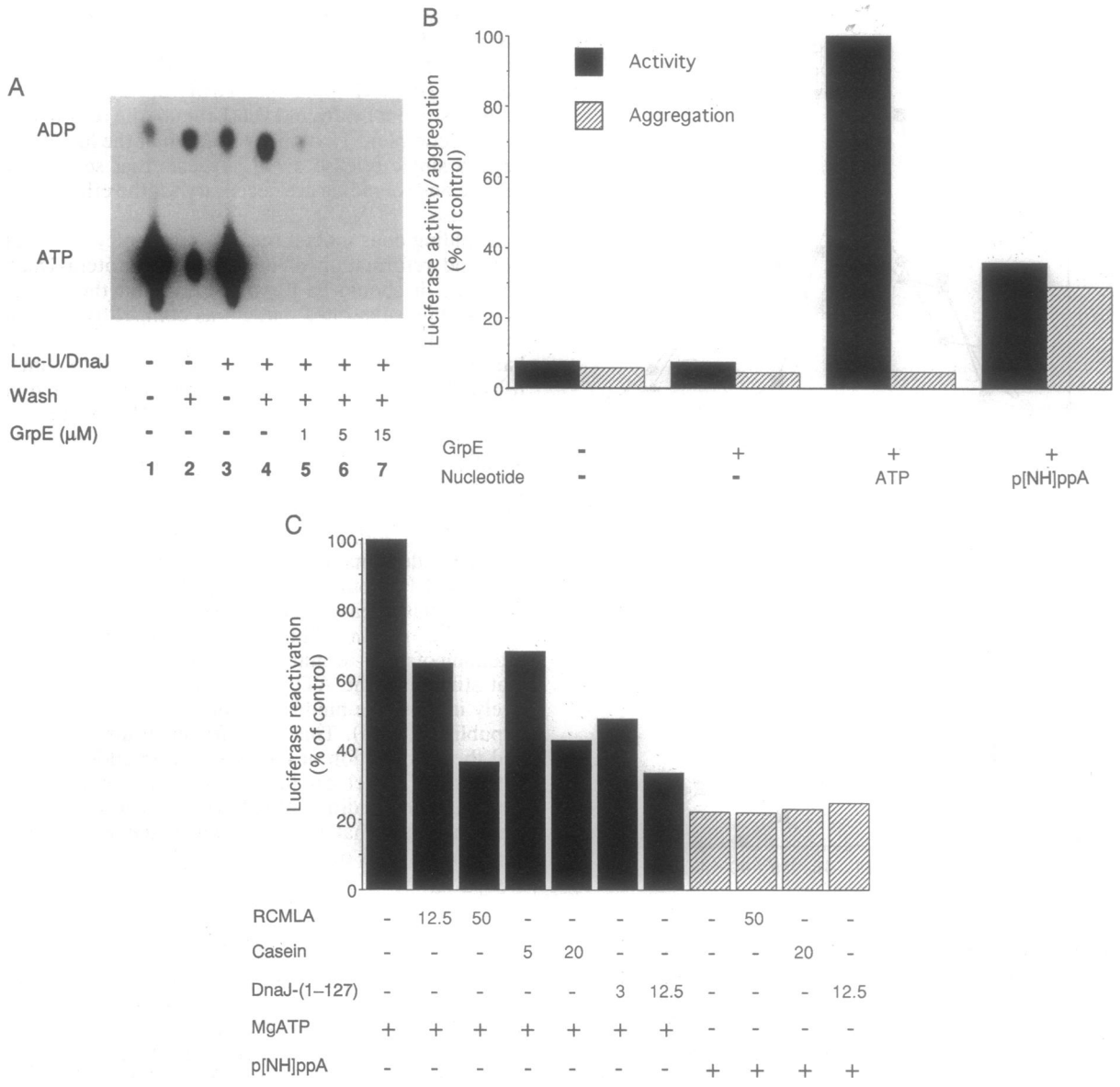

FIG. 3. Requirement for GrpE and nucleotide for the dissociation of substrate protein–DnaK–DnaJ complexes. (*A*) Thin layer chromatography analysis of DnaK-bound nucleotide after incubation with [α-^{32}P]ATP. Lanes: 1, nucleotide present in the total reaction mixture after incubation of DnaK alone; 2, nucleotide remaining in the same reaction mixture as in lane 1 after microfiltration to remove free nucleotide; 3, total reaction mixture containing DnaK, DnaJ, and denatured luciferase; 4–7, reaction mixture in lane 3 after incubation with the indicated concentrations of GrpE and microfiltration. In lanes 1 and 3, 10 times less sample was applied. (*B*) Percentage of luciferase reactivated (solid bars) or aggregated (hatched bars) from a luciferase–DnaK–DnaJ complex formed in the presence of MgATP and then separated from free nucleotide by microfiltration. The refolding reaction was initiated by addition of MgATP, p[NH]ppA, and GrpE as indicated. Percentage of luciferase aggregating was determined as the fraction that sedimented upon centrifugation. (*C*) Reactivation of luciferase from a reisolated luciferase–DnaK–DnaJ complex in the presence of reduced carboxymethylated α-lactalbumin (RCMLA) or α-casein, competitive inhibitors for rebinding to DnaK and DnaJ, respectively, or an N-terminal fragment of DnaJ (residues 1–127).

proceed rapidly to the native state. This allows for multiple rounds of refolding.

In the absence of nucleotide, DnaK may be in equilibrium between high- and low-affinity states for substrate binding. The reported slow association of unfolded proteins with nucleotide-free Hsp70s in the absence of DnaJ ($t_{1/2} \approx 10$ min) (10, 24) may thus reflect the rate of interconversion between these two states. Consistent with our model, such substrate–Hsp70 complexes have been shown to be dissociated by the addition of ATP (10, 22, 25, 26) and stabilized by ADP (10). This is also supported by the recent demonstration that the dissociation of substrate–DnaK complexes is too rapid to be accounted for by the slow ATP hydrolytic activity of DnaK (11, 12). Based on this observation, it has been proposed that the interaction between substrate proteins and DnaK does not involve a cycle of ATP hydrolysis (12). In contrast, our results indicate that DnaK fulfills a physiologically relevant function only in the context with a DnaJ- and GrpE-dependent cycle of ATP hydrolysis. Indeed, DnaK alone will not refold luciferase unless it is first bound by DnaJ (Fig. 1). However, in contrast to previous views (22, 27), we demonstrate that ATP hydrolysis by DnaK is required for the rapid formation of the polypeptide–DnaK interaction and not for its dissociation.

Recently, DnaJ has been shown to bind short ribosome-bound polypeptide chains (23). This and other findings (28) suggest that the early prevention of incorrect folding may in fact be mediated by DnaJ homologs in cooperation with Hsp70. In view of these observations, the question arises whether *in vivo* the Hsp70 system is sufficient to mediate the

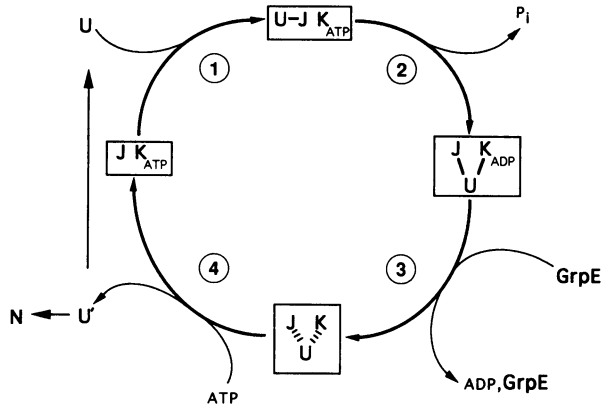

FIG. 4. Model for the mechanism of action of DnaK, DnaJ, and GrpE in the refolding of denatured luciferase. For the sake of simplicity, only a single molecule of DnaK and of DnaJ is shown to participate in the folding-competent complex with luciferase, although the exact stoichiometry of the complex is not yet known. U, unfolded protein; U', protein conformation of a fraction of luciferase molecules upon release from DnaK and DnaJ; N, native state.

folding of certain proteins or whether the major role of DnaK, DnaJ, and GrpE is to prevent aggregation and to transfer unfolded protein to the chaperonin GroEL–GroES for folding. Such a coupled reaction has been demonstrated for the mitochondrial protein rhodanese, which will not refold in the presence of DnaK, DnaJ, and GrpE alone (24). Luciferase may therefore be an example of a class of proteins that does not proceed through the complete pathway of chaperone-assisted folding. This would be supported by the observation that luciferase can be expressed in *E. coli* in active form (29), although GroEL is incapable of mediating the folding of luciferase *in vitro* (20). This raises a question concerning the nature of the coupling between the two chaperone systems *in vivo*. Whether transfer from DnaK and DnaJ to GroEL–GroES occurs may depend on the ability of the protein to fold rapidly upon release from DnaK–DnaJ. Alternatively, the two systems may be coupled directly so that transfer becomes independent of a protein's folding properties.

In addition to their role in protein folding, DnaK, DnaJ, and GrpE are also involved in other cellular processes, including the initiation of DNA synthesis from various phage and plasmid origins of replication (30, 31) and the autoregulation of heat shock gene transcription (32, 33). In these cases, the three proteins cooperate to induce changes in protein structure, for example, disassembling inactive dimers of the RepA initiator protein into active monomers (34). It would be expected that there is a common mechanism of substrate binding and release for the function of DnaK, DnaJ, and GrpE in protein folding and in these other systems.

We thank Drs. J. Rothman, R. Hlodan, J. Hendrick, and M. Mayhew for helpful discussions and for critically reading the manuscript. This work was supported by National Institutes of Health Grant GM48742.

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