DnaK, DnaJ, and GrpE heat shock proteins of Escherichia coli activate site-specific DNA binding by the RepA replication initiator protein of plasmid P1 in a reaction dependent on ATP and Mg$^{2+}$. We previously showed that GrpE is essential for in vitro RepA activation specifically at about 1 $\mu$M free Mg$^{2+}$. In this paper, we demonstrate that GrpE lowers the requirement of DnaK ATPase for Mg$^{2+}$, resulting in a large stimulation of ATP hydrolysis at about 1 $\mu$M Mg$^{2+}$ with and without DnaJ and RepA. In contrast to its effect on the Mg$^{2+}$ requirement, GrpE increases the ATP requirement for DnaK ATPase and dramatically lowers the affinity of DnaK for ATP in the absence of Mg$^{2+}$. We propose that GrpE not only lowers the affinity of DnaK for nucleotide but, by increasing affinity of DnaK for Mg$^{2+}$, also weakens the interactions of Mg$^{2+}$ with nucleotide prior to its release.

Molecular chaperones from the 70-kDa heat shock protein (Hsp70)$^{1}$ family are universally conserved ATPases, present in all prokaryotic and eukaryotic cells (1–3). Although the synthesis of Hsp70s is transiently stimulated in response to stress, most organisms synthesize them or related proteins, 70-kDa heat shock cognates (Hsc70s), constitutively in non-stress conditions. For normal growth, these molecular chaperones are required for folding newly synthesized polypeptides, translating polypeptides through membranes, proteolysis, disassembly of clathrin-coated vesicles, and DNA replication of several phage and plasmids. During heat shock, Hsp70s prevent aggregation of heat-inactivated proteins, dissociate protein aggregates formed by heat, and regulate the heat-shock response.

The most abundant Escherichia coli Hsp70 homolog, DnaK, acts with two other highly conserved heat shock proteins, DnaJ and GrpE. Mutations in dnaK, dnaJ, and grpE have similar pleiotropic effects on E. coli growth and physiology of several phage and plasmids. Furthermore, DnaK, DnaJ, and GrpE function together in several in vitro systems, including DNA replication of plasmid P1 and phage lambda (4–6) and reactivation of heat-inactivated RNA polymerase (7), luciferase (8), and rohdanese (9). In all of those systems, DnaJ tags selected native proteins or unfolded polypeptides for recognition by DnaK and stimulates the hydrolysis of DnaK-bound ATP. GrpE binds DnaK tightly through a conserved loop in the ATPase domain of DnaK near the nucleotide binding site (10) and releases nucleotides from DnaK (11). The stimulation of the nucleotide release from DnaK by GrpE results in increased DnaK ATPase activity and more efficient recycling of DnaK. Homologs of DnaJ and GrpE have been found in many eukaryotic and prokaryotic cells, suggesting that those two heat shock proteins are required for many, if not all, biological functions of Hsp70s and Hsc70s (12–14, 29).

In plasmid P1 replication DnaK, DnaJ, and GrpE activate site-specific DNA binding by the replication initiator protein, RepA (15, 16). RepA activation in vitro with purified proteins results from the conversion of RepA dimers to monomers (17–19). RepA dimers are inactive for specific DNA binding, but they form a complex with DnaJ dimers that then can be recognized by DnaK. DnaK in an ATP-dependent reaction dissociates the RepA-DnaJ complex such that RepA is released in a monomeric form. RepA monomers bind avidly to specific DNA sequences within the P1 plasmid origin. In vitro, the RepA activation reaction can be efficiently catalyzed by DnaJ and DnaK without GrpE (17, 18). We discovered that the requirement for GrpE emerges specifically when the free Mg$^{2+}$ concentration is lowered to about 1 $\mu$M (20). In this paper, we demonstrate that GrpE increases the affinity of DnaK ATPase for Mg$^{2+}$, resulting in the stimulation of both DnaK ATPase and RepA activation at about 1 $\mu$M Mg$^{2+}$. We show also that GrpE decreases the affinity of DnaK for ATP and discuss the possibility that the nucleotide release from DnaK by GrpE requires not only the lowering of DnaK affinity for nucleotide but also the weakening of the interactions of nucleotide with Mg$^{2+}$.

**MATERIALS AND METHODS**

Proteins and DNA—All solutions and buffers were prepared with MilliQ water and were purified on CHELEX-100 (Bio-Rad) (21). DnaK was purified by a procedure modified from that previously described (22). Following elution from ATP-agarose, DnaK was dialyzed against 60 ng of buffer A with 10% perchloric acid. DnaJ, DnaK, and RepA were purified as described (23). DnaK, DnaJ, and RepA were purified as described (20).

$[^{3}H]$HoriP1 DNA (an 1186-base pair DNA fragment containing five RepA binding sites in the P1 origin) was prepared as described (17).

**Assay for RepA Activation—**Reaction mixtures (20 $\mu$l) for RepA activation were assembled at 0°C and contained buffer A with 100 mM KCl, 20 $\mu$M ATP, 0.1 mM MgCl$_2$, metal chelators (as indicated), 60 ng of RepA, 60 ng of DnaK, 800 ng of DnaK, and 60 ng of GrpE (when indicated). After 5–20 min at 30°C, the mixtures were cooled to 0°C, and then $[^{3}H]$HoriP1 DNA fragment (50 fmol; 700 cpm/fmol) and calf thymus DNA (50 $\mu$g/ml) were added (17). After 5 min at 0°C, the mixtures were filtered through nitrocellulose filters (25-mm Schleicher and Schuell type BA85, 0.45 $\mu$m), and the retained radioactivity was measured.

**Assay for ATP Hydrolysis—**Reaction mixtures (20 $\mu$l) were assembled at 0°C without ATP and contained buffer A with 100 mM KCl, 0.2 mM EDTA, and 0.01–10 mM MgCl$_2$, as indicated. The mixtures were preincubated at 0°C for 10 min to stabilize the free Mg$^{2+}$ concentration,
and then DnaK, GrpE, and DnaJ were added as indicated. The reactions were initiated by the addition of 20 μM ATP with 0.1 μCi of \( \gamma^{32}P \)ATP (4500 Ci/mmol, ICN) and incubated at 30 °C. After 2–60 min, 2-μl samples were removed from the reaction mixtures and applied to PEI-cellulose thin-layer sheets. The \( \gamma^{32}P \) was separated from \( \gamma^{32}P \)ATP by thin layer chromatography using 1 M formic acid, 1 M LiCl (31). The chromatograms were cut in pieces, and the radioactivity was measured. For each reaction condition, at least five samples were taken in the linear range of the assay, and the amount of ATP hydrolysis was not permitted to exceed 10% of the amount of ATP initially present.

Equilibrium Dialysis for Measurements of Nucleotide Binding—Equilibrium dialysis was performed in an equilibrium microvolume dialyzer (EMD101B, Hoefer Scientific Instruments), which held 100 μl of solution on each side of the dialysis membrane. Nucleotide-free DnaK at concentrations of 0.02, 0.3, 3, and 20 μM, with or without equimolar amounts of GrpE, was dialyzed for 42 h at 4 °C, with 0.004–100 μM ATP and 0.1 μCi of \( \gamma^{32}P \)ATP (4500 Ci/mmol, from ICN) in 20 mM HEPES, pH 7.6, 100 mM NaCl, 100 mM KCl, 0.1 mg/ml bovine serum albumin, 5% glycerol, 5 mM dithiothreitol, and 20 mM EDTA. After the 42-h dialysis, less than 2% of the \( \gamma^{32}P \)ATP was hydrolyzed, and 70–90% of the ATP and 75–90% of the proteins were recovered depending on the initial concentration.

Free Mg\(^{2+}\) Calculation—The free Mg\(^{2+}\) concentration was calculated using the CHELATOR program (24) with the following conditions: 30 °C, 20 mM HEPES, pH 7.6, 20 μM ATP, 0.2 M ionic strength, 0.2 mM EDTA, and 0.02–10 mM MgCl\(_2\).

RESULTS

**GrpE Is Essential for P1 RepA Activation at About 1 μM Free Mg\(^{2+}\)**—To Increase the Affinity of DnaK ATPase for Mg\(^{2+}\)**—We observed previously that the specific DNA binding by the P1 RepA protein is activated in an ATP-dependent reaction by DnaK and DnaJ or DnaK, DnaJ, and GrpE, depending on the concentration of free Mg\(^{2+}\) in the reaction mixtures (20). With 100 μM Mg\(^{2+}\) and 20 μM ATP, GrpE is not required, but it becomes essential when the concentration of free Mg\(^{2+}\) is lowered to about 1 μM by EDTA or by physiological metal-ion buffers like citrate, phosphate, or pyrophosphate (Fig. 1A). We showed before that GrpE stimulates DnaK and DnaJ to activate RepA at 1 μM Mg\(^{2+}\) because it specifically lowers the free Mg\(^{2+}\) requirement for the RepA activation reaction (20).

Since Mg\(^{2+}\) is essential for DnaK ATPase activity, we thought that it was likely that the interplay of GrpE and Mg\(^{2+}\) may affect DnaK ATPase and measured rates of ATP hydrolysis directly in RepA activation conditions using the protein concentrations that were optimal for RepA activation. At about 1 μM free Mg\(^{2+}\), maintained by citrate, phosphate, or EDTA, the \( V_{\text{max}} \) of DnaK ATPase activity with RepA and DnaJ was lowered 4–6-fold (from 0.70 ± 0.05 pmol min\(^{-1}\) to 0.11–0.17 pmol min\(^{-1}\)) and, when maintained by pyrophosphate, 2-fold (to 0.32 ± 0.03 pmol min\(^{-1}\)). With these conditions, GrpE stimulated ATP hydrolysis by DnaK in the presence of RepA and DnaJ to 0.4–0.8 pmol min\(^{-1}\), nearly the \( V_{\text{max}} \) of DnaK alone (Fig. 1B). The stimulation of DnaK ATPase by GrpE in RepA activation conditions with 1 μM Mg\(^{2+}\) correlates nicely with the stimulation of the RepA activation reaction by GrpE. Moreover, GrpE lowered the Mg\(^{2+}\) requirement for ATP hydrolysis by DnaK in RepA activation conditions from 8 to 1 μM to 1.5 ± 0.5 μM (Fig. 2), similar to its lowering of the Mg\(^{2+}\) requirement for RepA activation (20). The observations that GrpE stimulates both DnaK ATPase and RepA activation at 1 μM Mg\(^{2+}\) and lowers the Mg\(^{2+}\) requirement for both ATP hydrolysis and RepA activation suggest that GrpE acts in RepA activation in vitro to facilitate the utilization of Mg\(^{2+}\) by DnaK ATPase and to stimulate ATP hydrolysis.

GrpE Lowers the Requirement of DnaK ATPase for Mg\(^{2+}\)**—Independently from RepA Activation—We wanted to characterize more precisely the effect of GrpE on the requirement of DnaK ATPase for Mg\(^{2+}\) in the presence and absence of DnaJ and determine whether this function of GrpE is general or limited to the in vitro RepA activation reaction.

The maximal velocity of 0.66 μM DnaK ATPase alone with 20 μM ATP and 10 mM Mg\(^{2+}\) was 0.60 ± 0.03 pmol min\(^{-1}\) at 30 °C, and the turnover number was 0.05 ± 0.003 min\(^{-1}\), similar to values previously reported (25). With EDTA as a metal ion buffer, the Mg\(^{2+}\) titration curve was sigmoidal, and half-maximal ATPase required a free Mg\(^{2+}\) concentration of 9 ± 1 μM Mg\(^{2+}\) (Fig. 3). We obtained a similar Mg\(^{2+}\) requirement with a 5-fold higher DnaK concentration and in conditions without EDTA (data not shown). The sigmoidal shape of the Mg\(^{2+}\) titration curve suggests some Mg\(^{2+}\)-dependent cooperativity in the function of DnaK ATPase.

We observed that GrpE in combination with DnaJ lowered the Mg\(^{2+}\) concentration required for half-maximal DnaK ATPase about 10-fold, from 9 ± 1 to 1.5 ± 0.5 μM (Fig. 4). In

**FIG. 1.** GrpE effects on RepA activation by DnaK and DnaJ, and on ATP hydrolysis by DnaK in the presence of DnaJ and RepA, with and without Mg\(^{2+}\)-chelating agents. Reaction mixtures were as described under “Materials and Methods” for RepA activation and contained the following: no chelators (None), 20 mM citrate (Citrate), 16 mM phosphate (Pi), 0.6 mM pyrophosphate (PPi), or 0.15 mM EDTA (EDTA) as indicated. A, RepA activation with GrpE (black bars) or without GrpE (gray bars) was measured as described under “Materials and Methods.” B, ATP hydrolysis was measured in RepA activation conditions containing 0.1 μCi of \( \gamma^{32}P \)ATP (4500 Ci/mmol) with GrpE (black bars) or without GrpE (gray bars) as described under “Materials and Methods.”

**FIG. 2.** Effect of GrpE on the free Mg\(^{2+}\) requirement for ATP hydrolysis in RepA activation. ATP hydrolysis was measured directly in RepA activation conditions containing 0.1 μCi of \( \gamma^{32}P \)ATP (4500 Ci/mmol) and 0.15 mM EDTA, with GrpE ( ) or without GrpE (○).
GrpE Alters the Affinity of DnaK for ATP and Mg$^{2+}$

The stimulation of the nucleotide exchange from DnaK was proposed to be the major function of GrpE, which directly or indirectly controls the binding and release of polypeptide substrates from DnaK (11, 26, 30). Despite its importance, little is known about the mechanism by which the association of GrpE with DnaK releases the nucleotide and by which ATP can be utilized. We used 0.6 µM DnaK, the DnaK concentration required for RepA activation, and measured the rate of ATP hydrolysis at various concentrations of ATP. We found that with these conditions, GrpE increased the ATP requirement for half-maximal DnaK ATPase from 1 ± 0.1 µM to 2.6 ± 0.5 µM (Fig. 7). Therefore, in conditions where GrpE increases the affinity of DnaK for Mg$^{2+}$, thus facilitating the utilization of Mg$^{2+}$ for DnaK ATPase, it simultaneously lowers the affinity of DnaK for ATP.

Without Mg$^{2+}$, GrpE increased the $K_a$ for ATP binding by DnaK by 100-fold, from about 0.5 µM to at least 100 µM, as measured directly by equilibrium dialysis experiments (Fig. 8). Therefore, GrpE lowers the affinity of DnaK for nucleotide more dramatically without than with Mg$^{2+}$.

**DISCUSSION**

The stimulation of the nucleotide exchange from DnaK was found to be the major function of GrpE. Our finding that GrpE increases the affinity of DnaK for Mg$^{2+}$ suggested that GrpE may also increase the affinity of DnaK for nucleotide complexed with Mg$^{2+}$. This possibility was puzzling in light of the nucleotide exchange function of GrpE. To solve this dilemma, we measured the effect of GrpE on the ATP requirement for DnaK ATPase in conditions with about 1 µM free Mg$^{2+}$ (100 µM Mg$^{2+}$ and 150 µM EDTA), where DnaK ATPase depends on GrpE for Mg$^{2+}$ utilization. We used 0.6 µM DnaK, the DnaK concentration required for RepA activation, and measured the rate of ATP hydrolysis at various concentrations of ATP. We found that with these conditions, GrpE increased the ATP requirement for half-maximal DnaK ATPase from 1 ± 0.1 µM to 2.6 ± 0.5 µM (Fig. 7). Therefore, in conditions where GrpE increases the affinity of DnaK for Mg$^{2+}$, thus facilitating the utilization of Mg$^{2+}$ for DnaK ATPase, it simultaneously lowers the affinity of DnaK for ATP.

Without Mg$^{2+}$, GrpE increased the $K_a$ for ATP binding by DnaK by 100-fold, from about 0.5 µM to at least 100 µM, as measured directly by equilibrium dialysis experiments (Fig. 8). Therefore, GrpE lowers the affinity of DnaK for nucleotide more dramatically without than with Mg$^{2+}$.
multaneously higher ATP concentration. The additional observation that GrpE lowers the affinity of DnaK for ATP much more dramatically without than with Mg$^{2+}$ suggests the possibility that nucleotide release requires not only the lowering of the affinity of DnaK for nucleotide but also the weakening of the Mg$^{2+}$-nucleotide interactions. This may be accomplished by a rearrangement of the negatively charged amino acid residues ligating Mg$^{2+}$ in the active center of DnaK ATPase such that Mg$^{2+}$ would be at least transiently bound by DnaK with an increased affinity. The crystallographic analysis of the structure of the ATPase fragment of bovine brain Hsc70 supports the possibility that rearrangements of residues Asp-10 and Glu-175 may result in strengthening the ligation of Mg$^{2+}$ (27). Moreover, it has been shown that GrpE efficiently releases ATP from DnaK prior to ATP hydrolysis (11).

We have demonstrated that GrpE lowers the Mg$^{2+}$ requirement for DnaK ATPase both with RepA activation conditions and with DnaK ATPase conditions. This suggests that the increased DnaK affinity for Mg$^{2+}$ observed with GrpE is important for hydrolysis of ATP bound to the DnaK-GrpE complex. This observation also brings into question whether or not the stimulation of DnaK ATPase by GrpE is due solely to its nucleotide exchange function. It is possible that the effect of GrpE on the ligation of Mg$^{2+}$ contributes to the stimulation of DnaK ATPase, especially in conditions with high concentrations of ATP when the inhibition by ADP and P$_i$ products is negligible.

The control of GrpE over DnaK amino acid residues ligating Mg$^{2+}$ may also be important for discrimination against the binding of other divergent metal ions to the active site of DnaK ATPase. The precise position and electrostatic environment of Mg$^{2+}$ in the active center of Hsc70 ATPase was suggested to be critical for the ATPase activity, based on the observation that all the residues ligating Mg$^{2+}$ are highly conserved and cannot be changed without affecting ATPase activity (27, 28). Such a high conservation of the Mg$^{2+}$ ligands in the ATPase active site suggests that Hsp70 may be very selective in its requirement for Mg$^{2+}$ and sensitive for any rearrangements of the Mg$^{2+}$ environment. Contrary to that, Hsp70s are functional even in the presence of heavy metals that stimulate the heat shock response. The GrpE-dependent discrimination of other metal ions from binding to DnaK is an intriguing hypothesis that provides a link between the specific requirement for GrpE observed in the in vitro RepA activation with DnaK and DnaJ to utilize Mg$^{2+}$ for DnaK ATPase at low Mg$^{2+}$ concentration and the requirement for GrpE in vivo, likely to facilitate the utilization of Mg$^{2+}$ in the presence of other metal ions competing for binding to DnaK.

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