Physical Interaction between Bacterial Heat Shock Protein (Hsp) 90 and Hsp70 Chaperones Mediates Their Cooperative Action to Refold Denatured Proteins*

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Background: HtpG, a bacterial heat shock protein 90 (Hsp90), is essential for thermotolerance in some prokaryotes.
Results: HtpG functions with DnaK2/DnaJ2/GrpE to assist unfolding/folding of denatured proteins in both ATP-dependent and -independent fashions.
Conclusion: The cooperative action of HtpG and DnaK2 might play a key role under stress.
Significance: This might be the first sign of a prokaryotic Hsp90 foldosome.

In eukaryotes, heat shock protein 90 (Hsp90) is an essential ATP-dependent molecular chaperone that associates with numerous client proteins. HtpG, a prokaryotic homolog of Hsp90, is essential for thermotolerance in cyanobacteria, and *in vitro* it suppresses the aggregation of denatured proteins efficiently. Understanding how the non-native client proteins bound to HtpG refold is of central importance to comprehend the essential role of HtpG under stress. Here, we demonstrate by yeast two-hybrid method, immunoprecipitation assays, and surface plasmon resonance techniques that HtpG physically interacts with DnaJ2 and DnaK2. DnaJ2, which belongs to the type II J-protein family, bound DnaK2 or HtpG with submicromolar affinity, and HtpG bound DnaK2 with micromolar affinity. Not only DnaJ2 but also HtpG enhanced the ATP hydrolysis by DnaK2. Although assisted by the DnaK2 chaperone system, HtpG enhanced native refolding of urea-denatured lactate dehydrogenase and heat-denatured glucose-6-phosphate dehydrogenase. HtpG did not substitute for DnaJ2 or GrpE in the DnaK2-assisted refolding of the denatured substrates. The heat-denatured malate dehydrogenase that did not refold by the assistance of the DnaK2 chaperone system alone was trapped by HtpG first and then transferred to DnaK2 where it refolded. Dissociation of substrates from HtpG was either ATP-dependent or -independent depending on the substrate, indicating the presence of two mechanisms of cooperative action between the HtpG and the DnaK2 chaperone system.

The 90-kDa heat shock proteins (Hsp90s) are a family of molecular chaperones widespread in eubacteria and eukaryotes. Proteomic analysis shows that Hsp90s are the most abundant proteins in human cell cultures, which may reach up to 2% of the total protein mass (1). Hsp90 is conserved from bacteria to eukaryotes with ∼50% sequence similarity between *Escherichia coli* and humans. It forms a homodimer through the interaction of the C-terminal domain. Hsp90 has a very low ATPase activity causing large conformational changes of Hsp90 that are thought to drive structural changes in a bound substrate protein and mediate its subsequent release from the chaperone (2–5).

In eukaryotes, Hsp90 functions with various substoichiometric co-chaperones such as Hop/Sti1, Cdc37, Aha1, and p23/Sba1. They transiently associate with Hsp90 to physically control the ATPase activity of Hsp90 and/or to recruit specific substrate proteins. Hop/Sti1 also mediates the interaction between Hsp90 and Hsp70. It binds simultaneously to Hsp90 and Hsp70 through the interaction of its multiple tetratricopeptide repeat domains with the C-terminal motifs of Hsp90 and Hsp70. Hop/Sti1 regulates the function of both chaperones and facilitates substrate protein transfer from Hsp70 to Hsp90 (2–6). Like other major classes of heat shock proteins, such as Hsp60 and Hsp70, Hsp90 may also be described as a "holding" chaperone by virtue of its ability *in vitro* to recognize and bind non-native proteins and prevent their aggregation (7).

Relatively little is known about high temperature protein G (HtpG), which is the term for the prokaryotic homolog of Hsp90. Whereas eukaryotes require a functional cytoplasmic Hsp90 for viability under all conditions (8), the bacterial HtpG proteins are not required under normal growth conditions and even under heat stress in heterotrophic bacteria, such as *E. coli* and *Bacillus subtilis* (9, 10). No co-chaperones/cofactors for HtpG have been detected.

Cyanobacteria are oxygenic photosynthetic prokaryotes that are phylogenetically related to chloroplasts of photosynthetic

eukaryotes. As in other prokaryotes, the expression of HtpGs in cyanobacteria such as *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803 is highly induced upon transfer to high temperature, but in contrast to heterotrophic bacteria, mutation of the *htpG* gene impairs growth and/or survival of the two cyanobacterial strains greatly under heat stress, although HtpG is nonessential under normal conditions (11, 12). Not only high temperature but also low temperature and oxidative stresses cause impaired phenotype of the *Synechococcus htpG* mutant (13, 14). The importance of HtpG under low temperature and oxidative stresses in other bacteria, such as psychrophilic and pathogenic bacteria, has also been reported (15, 16). Protein substrates for cyanobacterial and *E. coli* HtpG have been identified (17–19), but it is not known by what mechanism HtpG plays a role under stress at the molecular level. Recently, it was shown *in vitro* that the *E. coli* HtpG functions with the DnaK chaperone system to refold a denatured substrate. The DnaK system consists of DnaK, GrpE, and DnaJ or CbpA. Their results suggest that the DnaK chaperone system acts first on the substrate protein, and then HtpG and the DnaK system function collaboratively to complete remodeling of the substrate (20).

In this study, we showed that HtpG is not capable of assisting refolding of a denatured substrate by itself, but it can function cooperatively with the DnaK system to assist unfolding/folding of a denatured substrate in both ATP-dependent and ATP-independent fashions. HtpG could bind and form a soluble complex with a denatured substrate that was transferred in an ATP-independent fashion to the DnaK system where the substrate refolded. In the case of a substrate that does not form a large aggregate, but is unable to refold easily only by the assistance of the DnaK system, it was transferred to HtpG. HtpG assisted its refolding in an ATP-dependent fashion.

EXPERIMENTAL PROCEDURES

Construction of Overexpression Vectors/Strains and Purification of His-tagged Proteins—The coding regions of *dnaK2* (NCBI-GI:507819) and *grpE* (NCBI-GI:81169762) were PCR-amplified from *S. elongatus* PCC 7942 chromosomal DNA with pairs of oligonucleotide primers 5′-AACATATGGCCAAA-GTTGTCGGA-3′ and 5′-AAGGATCCTTCGACTCAGAGA-ACTC-3′, and 5′-AACATATGAGCGAACATCAGACC-3′ and 5′-AAAAGCTTTTCTGTTACCGCTGCAGA-3′, respectively. The primers contained an NdeI, BamHI, or HindIII restriction site for cloning (underlined in the above primer sequences). Each PCR fragment was cloned into pT7Blue T-Vector (Novagen), digested with the respective restriction enzymes, and subcloned into pET-21a vector (Novagen) digested with the same enzymes. Construction of an overexpression vector for the C-terminally His-tagged HtpG has been described previously (18). The constructed plasmids were transformed into *E. coli* BL21 (DE3). When expressed, all the translation products carried a C-terminally fused His_6 tag. The *E. coli* cells that overexpressed DnaK2 or GrpE were harvested, suspended in 20 mM NaH_2PO_4/Na_2HPO_4 buffer at pH 8.0 containing 500 mM NaCl (lysis buffer), and disrupted by sonication. The soluble cell extract was loaded onto a column of nickel-chelated Sepharose 6 Fast Flow (GE Healthcare) that had been equilibr-

ated with the lysis buffer. After washing the column with the lysis buffer containing 20 mM imidazole or 20 mM NaH_2PO_4/-Na_2HPO_4 buffer (pH 8.0) containing 1.5 M NaCl, 40 mM imidazole, and 1% Triton X-100, His-tagged proteins were eluted with the lysis buffer containing 500 mM imidazole. Immediately after the elution, purified proteins were dialyzed against 50 mM Hepes-KOH (pH 7.5). Construction of an overexpression vector for the N-terminally His-tagged DnaJ2 has been described previously (21). 1 mM DTT was added in all the buffers used for extraction and purification of the DnaJ2. Single substitution mutations of HtpG (E34A and D79N) were introduced into pET-HtpG, using the QuikChange mutagenesis system (Stratagene) as described by the manufacturer.

Yeast Two-hybrid Analysis—Yeast two-hybrid analysis for the *S. elongatus* PCC 7942 genomic library screening was carried out according to the method described previously (21). Positive protein-protein interaction between the bait and the prey was detected by the ability of the cells to grow on a synthetic complete (SC) plate lacking Leu, Trp, and adenine.

Immunoprecipitation Assays—Protein G-Sepharose 4 Fast Flow beads (GE-Healthcare) with coupled *Synechococcus vulcanus* DnaK or *S. elongatus* PCC 7942 HtpG polyclonal antibodies (13, 22), which were pre-equilibrated in phosphate-buffered saline, were incubated with DnaJ2 (1.0 nmol) and/or DnaK2 (1.0 nmol) or DnaJ2 (1.0 nmol) and/or HtpG (1.0 nmol) for 2 h at 4 °C. Beads were washed three times with phosphate-buffered saline containing 0.05% Tween 20 and heated at 100 °C for 3 min in SDS-PAGE sample buffer containing 60 mM Tris-HCl (pH 6.8), 6% (w/v) sucrose, 2% (w/v) SDS, 0.03% (w/v) bromophenol blue, and 60 mM dithiothreitol. After centrifugation, the supernatant fraction was applied for SDS-PAGE.

Interaction Analyses on CM5 Sensor Chip Using Surface Plasmon Resonance (SPR)—To analyze the interactions between DnaJ2, HtpG, and DnaK2, we used SPR[2] analyses on CM5-chip using Biacore T100 (GE Healthcare). For the DnaJ2-HtpG interaction analyses, 100 nM DnaJ2 was immobilized on activated flow cell 2 of CM5 chip through an amino-coupling reaction, followed by the blocking of the unreacted carboxyl groups with ethanolamine-HCl. Once the surface of CM5-chip was prepared, we injected HtpG as an analyte into the flow cells 1 (control surface) and 2, at a flow rate of 30 μl/min for 2 min. Binding data sets of five different concentrations of analyte were collected using multicycle mode. The binding data were analyzed using Biacore T100 evaluation software 2.0.2 version (GE Healthcare) and fitted with 1:1 binding model. The fitting data were displayed with global rate constants (association rate k_a, dissociation rate k_d, and equilibrium dissociation constant K_D). Similarly, the DnaJ2-DnaK2 and DnaK2-HtpG interactions were evaluated as mentioned above. In these cases, we immobilized DnaJ2 and DnaK2 as ligands and injected DnaK2 and HtpG as analytes, respectively. All binding measurements were carried out in HBS-P$^+$ buffer (10 mM Hepes, 150 mM NaCl, 0.05% (v/v) surfactant P20 (pH 7.4)).

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[2] The abbreviations used are: SPR, surface plasmon resonance; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase.

ATPase Activity Measurements—Steady-state ATPase activity of DnaK2 or HtpG was measured at 37 °C with an ATP regeneration system. The assay mixture (1 ml) consisted of 50 mM Hepes-KOH (pH 7.5), 6 mM Mg(CH$_3$COO)$_2$, 20 mM KCl, 2 mM phosphoenolpyruvate, 30 μg of pyruvate kinase (PK) from rabbit muscle (Oriental Yeast), 200 μM NADH, 50 μg of rabbit muscle lactate dehydrogenase (LDH, Oriental Yeast), 100 μM ATP, DnaK2, DnaJ2, GrpE, and/or HtpG. ATP hydrolysis was initiated by the addition of ATP to the reaction mixture. The decrease in the absorbance at 340 nm was monitored continuously with the Shimadzu UV-1600PC spectrophotometer (Shimadzu).

Anti-aggregation Assay (Light Scattering Measurements)—50 mM Hepes-KOH (pH 7.5) containing 5 mM DTT (1 ml) was preincubated at 45 °C for 5 min. Then, porcine heart mitochondrial malate dehydrogenase (MDH) purchased from Sigma and/or a chaperone/co-chaperone (HtpG, DnaJ2, or DnaK2) protein was added to the solution, and prevention of aggregation of MDH was assayed at 45 °C by continuous monitoring of the absorbance at 360 nm.

LDH Refolding Assay—Denaturation of 18.75 μM LDH was carried out in 50 mM Hepes-KOH (pH 7.5) containing 5 M urea and 5.0 mM DTT for 10 min at 25 °C. To start a refolding assay, 74 μl of refolding solution was added to 1 μl of the denatured LDH solution at 25 °C. Thus, the final concentration of LDH was 0.25 μM after the dilution. The refolding solution contained 50 mM Hepes-KOH (pH 7.5), 20 mM Mg(CH$_3$COO)$_2$, 150 mM KCl, 10 mM phosphoenolpyruvate, 40 μg/ml PK, 10 mM DTT, 4 mM ATP, 6 μM DnaK2, 1.2 μM DnaJ2, and 0.6 μM GrpE. Activity was assayed in a solution containing 50 mM Hepes-KOH (pH 7.5), 1 mM DTT, 10 mM pyruvate, and 200 μM NADH. To evaluate spontaneous refolding (chaperone-independent refolding) of the denatured enzyme, bovine serum albumin (BSA) was added in the same amount (in grams of protein) as the DnaK2 chaperone system (DnaK2/DnaJ2/GrpE).

Glucose-6-phosphate Dehydrogenase (G6PDH) Refolding Assay—Denaturation of 0.5 μM G6PDH from *Leuconostoc mesenteroides* (Oriental Yeast) was carried out in 50 mM Hepes-KOH (pH 7.5) containing 150 mM KCl, 20 mM Mg(CH$_3$COO)$_2$, and 10 mM DTT for 10 min at 52 °C. To measure the refolding of G6PDH, 30 μl of the double-concentrated refolding solution as described above was added to 30 μl of the denatured G6PDH solution at 25 °C. Activity was assayed in a solution containing 50 mM Tris-HCl (pH 7.8), 3 mM MgCl$_2$, 2 mM NAD$^+$, and 3.3 mM glucose 6-phosphate. The increase in the absorbance at 340 nm was monitored continuously.

MDH Refolding Assay—MDH (0.5 μM) was denatured at 45 °C for 30 min in a solution containing 50 mM Hepes-KOH (pH 7.5) in the presence of HtpG (1.0 μM) or the same amount (in grams of protein) of BSA as that of HtpG. To measure the MDH refolding, 50 μl of the double-concentrated refolding solution containing 10 μM DnaK2, 20 μM DnaJ2, and 10 μM GrpE was added to 50 μl of denatured MDH solution at 25 °C. To evaluate spontaneous refolding (chaperone-independent refolding) of the denatured enzyme, BSA was added in the same amount (in grams of protein) as that of the DnaK2/DnaJ2/GrpE chaperone system. MDH activity was measured by monitoring the oxidation of NADH at 340 nm in a solution containing 150
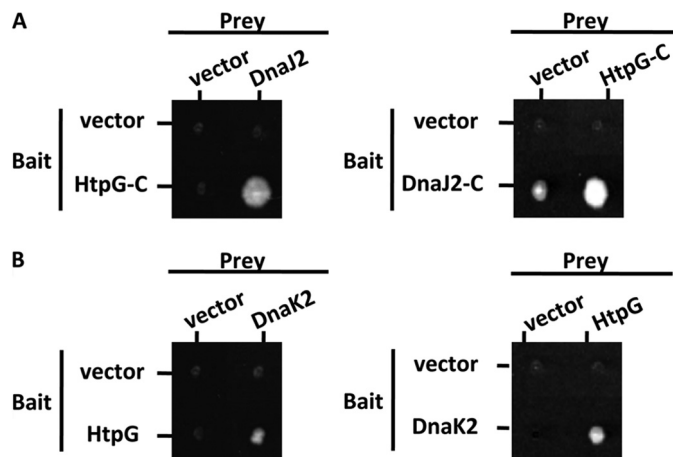

FIGURE 1. **Two-hybrid interactions of HtpG with DnaJ2 and DnaK2.** Yeast cells were grown for 1 week at 30 °C on an SC plate lacking leucine, tryptophan, and adenine. Diploid strains were constructed by mating PJ69-4Aa cells harboring pGBTK or its derivatives (*bait*) with PJ69-4Aa cells harboring pGAD424 or its derivatives (*prey*), where each derivative plasmid contained various fragments indicated in the figure. *HtpG-C* indicates a C-terminal half of HtpG (amino acids 344–639).

mM potassium phosphate buffer (pH 7.5), 10 mM DTT, 0.5 mM oxaloacetic acid, and 280 μM NADH.

RESULTS

Identification of Interaction of HtpG with DnaJ2 and DnaK2 by a Yeast Two-hybrid Screening—To elucidate a protein(s) that interacts with HtpG, we adopted a yeast two-hybrid screening with the *Synechococcus* genomic library (17). The full-length *htpG* gene or its C-terminal half (HtpG-C, amino acids 344–639) was cloned into pGBTK to obtain a bait clone. HtpG-C lacks the whole N-terminal domain that binds ATP. A large number of independent positive clones were obtained with HtpG-C. After sequencing a number of prey cloned into pGAD, we categorized them into fragments derived from a corresponding gene. Among them, we identified fragments of DnaJ2 (Synpcc7942_1789). These fragments (amino acids 66–326, and amino acids 100–326 (DnaJ2-C)) contain the C-terminal region but lack the J domain. HtpG-C also interacted with the full-length DnaJ2 (Fig. 1*A*). The interaction was also detected when the bait and prey constructs were switched. DnaJ2 also interacts with DnaK2 specifically (21). In addition to DnaJ2, we examined an interaction between HtpG and DnaK2 by the yeast two-hybrid system. The full-length HtpG interacted with the full-length DnaK2 (Fig. 1*B*). The interaction was also detected when the bait and prey constructs were switched.

There are three DnaK homologs, DnaK1, DnaK2, and DnaK3, and at least four J-proteins (J-domain proteins) in *S. elongatus* PCC 7942. DnaK2 and DnaJ2 are the only stress-inducible ones among the DnaK homologs and J-proteins (23, 24). HtpG is also stress-inducible (11, 13, 14). DnaJ2 is a type II J-protein, which is at variance with *E. coli* DnaJ but similar to *E. coli* CbpA (see Fig. 7*A*) that lacks the so-called Zn-binding domain (25). We could not detect any interactions between HtpG and the other J-domain containing proteins by the yeast two-hybrid screening, indicating that HtpG interacts with DnaJ2 specifically.
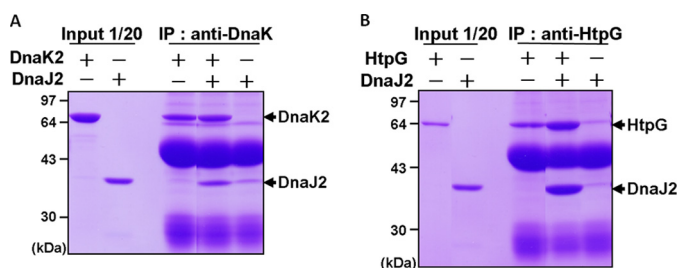
FIGURE 2. Physical interaction of DnaJ2 with DnaK2 (*A*) or HtpG (*B*). For immunoprecipitation (*IP*) assays, a mixture containing 1.0 nmol of DnaJ2 and/or 1.0 nmol of DnaK2 or a mixture containing 1.0 nmol of DnaJ2 and/or 1.0 nmol of HtpG was incubated at 4 °C for 2 h in the presence of protein G-Sepharose 4 Fast Flow beads with coupled DnaK2 or HtpG antibodies. Proteins co-precipitated with the beads were separated by SDS-PAGE (12%) and stained with Coomassie Brilliant Blue. As references, 0.05 nmol of DnaJ2, DnaK2, and HtpG was analyzed in the same gel and is shown on the *left*. DnaJ2, DnaK2, and HtpG are indicated by *arrows*. The largest band located in the middle of the gels is the heavy chain of IgG.

Physical Interaction of DnaJ2 with DnaK2 and HtpG Confirmed by Immunoprecipitation Assays—We purified HtpG, DnaK2, and DnaJ2, all of which were derived from the cyanobacterium *S. elongatus*. The purity of HtpG, DnaK2, and DnaJ2 was 98, 96, and 96%, respectively, when the density of SDS-polyacrylamide gel bands was determined using ImageJ (rsb. info.nih.gov). All the recombinant proteins except DnaJ2 were His-tagged at their C-terminal ends, whereas DnaJ2 was N-terminally His-tagged. Physical interaction between these isolated chaperones and co-chaperone was examined by immunoprecipitation assays using antibodies raised against HtpG or DnaK2. HtpG as well as DnaK2 was precipitated with DnaJ2 (Fig. 2). Physical interaction between HtpG and DnaK in other bacteria has been shown by immunoprecipitation assays (15).

Quantification of the Physical Interaction between HtpG, DnaK2, and DnaJ2 by SPR Technique—The interaction between HtpG, DnaJ2, and DnaK2 was quantified by the SPR technique. The K_D value for the HtpG-DnaJ2 complex was 102 nM (Fig. 3*A* and Table 1), although that for the DnaK2-DnaJ2 complex was 173 nM (Fig. 3*B* and Table 1). Thus, DnaJ2 showed similar or slightly higher affinity toward HtpG than DnaK2. DnaK2 interacted with HtpG with the K_D value of 2.58 μM (Fig. 3*C* and Table 1). Thus, HtpG interacted with DnaJ2 much more strongly than DnaK2.

Effect of HtpG on the ATPase Activity of DnaK2—It is well established that DnaJ/Hsp40 or J-protein interacts physically with DnaK/Hsp70 to enhance its ATPase activity, which is essential for chaperone function of DnaK (25). The cyanobacterial DnaJ2 that interacts physically with DnaK2 also enhanced the ATPase activity of DnaK2 (Fig. 4*A*). However, GrpE did not enhance the DnaK2 activity by itself, but it did when DnaJ2 was present as expected from the ATP-independent, ADP release activity of this co-chaperone (26).

We further examined the effect of HtpG on the ATPase activity of DnaK2 because we detected a physical interaction between the two proteins. The activity in the presence of HtpG was higher than that resulting from a simple addition of the two chaperone's activities measured separately, indicating that HtpG or DnaK2 enhances the ATPase activity of the other one (Fig. 4*B*). To measure only the DnaK2 activity, the ATPase activity of HtpG was specifically blocked by radicicol (3 μM), a

potent inhibitor of Hsp90/HtpG (27). No detection of the activity in the presence of the inhibitor indicated negligible contamination in the HtpG sample by other ATP-hydrolyzing enzymes. HtpG still enhanced the ATPase activity of DnaK2 in the presence of radicicol (Fig. 4*B*). HtpG doubled the ATPase activity of DnaK2 under our specific experimental conditions in the presence or absence of radicicol. The results indicate that HtpG enhances the ATPase activity of DnaK2, but not reciprocally. As far as we know, there is no report that HtpG/Hsp90 enhances the ATPase activity of DnaK/Hsp70.

We thought that the enhancement of the DnaK2 ATPase activity is due to the formation of the HtpG-DnaK2 complex. Thus, we examined the effect of varied HtpG concentrations on the enhancement. Within the concentration range examined in this study, as the concentration of HtpG increased, the more DnaK2 ATPase activity was enhanced (Fig. 4*C*). The enhancement did not saturate up to 5 μM HtpG, which is consistent with the K_D value of 2.58 μM for the DnaK2-HtpG complex (Table 1).

Effect of HtpG on the Folding Activity of DnaK2—It is well established that the chaperone function is coupled with the ATPase cycle of DnaK (25). Increase in the ATPase activity of DnaK2 by HtpG suggests that HtpG may enhance the DnaK2-mediated refolding of a denatured protein by increasing the ATPase activity of DnaK2. To test this, we first established the DnaK2/DnaJ2/GrpE chaperone system. There is no report about protein folding assisted by a cyanobacterial DnaK chaperone system. As shown below, when the chaperone and co-chaperones were added to a heat-denatured or a urea-denatured model protein substrate, the substrate refolded, as revealed by the recovery of its enzymatic activity. All the experiments with G6PDH, LDH, and MDH confirmed that the refolding was strictly dependent on the presence of the (co-)chaperones and ATP. ADP did not replace ATP. Individual members of the chaperone system did not result in refolding of the above enzymes under the experimental conditions used in this study. Both DnaK2 and its co-chaperone DnaJ2 were essential for denatured proteins to refold. The rate of the refolding was further enhanced by the addition of GrpE to a reaction mixture containing DnaK2 and DnaJ2 (Fig. 5*A*).

First, we tested whether HtpG can substitute for DnaJ2 or GrpE as a co-chaperone for DnaK2. As shown in Fig. 4, HtpG as well as DnaJ2 and GrpE enhanced the ATPase activity of DnaK2. G6PDH that had been heat-treated at 52 °C retained less than 5% of the initial activity, but it recovered about 50% of the activity when the enzyme solution was incubated at 25 °C in the presence of the DnaK2/DnaJ2/GrpE chaperone system. HtpG did not substitute for GrpE as a co-chaperone because there was no effect of HtpG on the refolding reaction in the presence of DnaK2 and DnaJ2 (Fig. 5*A*). HtpG did not substitute for DnaJ2 as a co-chaperone because there was no enhancement of refolding in the presence of DnaK2 and GrpE (Fig. 5*B*). However, when HtpG was added together with the DnaK2/DnaJ2/GrpE chaperone system, the rate of the refolding reaction significantly increased (Fig. 5*C*). These results indicate that the function of HtpG in the enhancement of the refolding is distinct from the co-chaperoning functions of DnaJ2 or GrpE. We tested by inhibiting the ATPase activity of
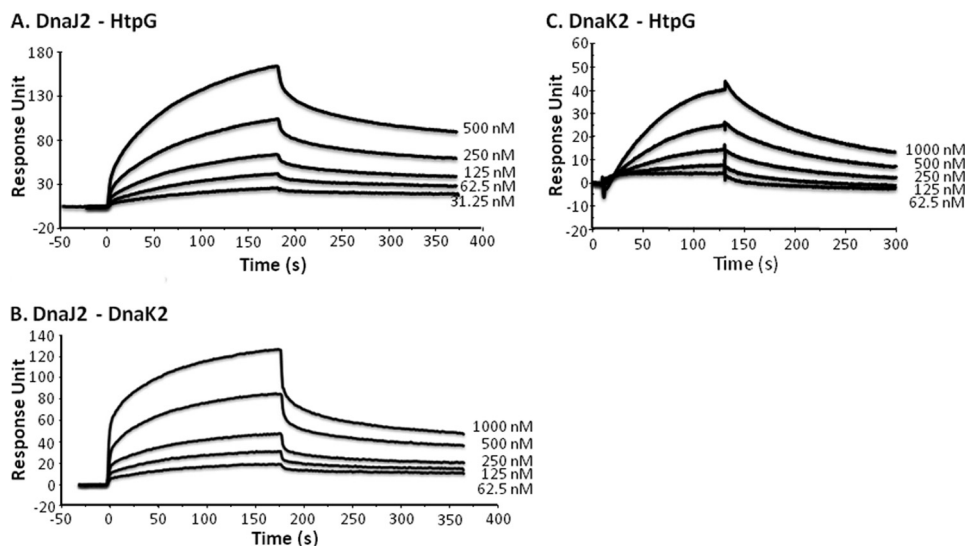
FIGURE 3. **Kinetic analysis of chaperone-chaperone/co-chaperone interactions with surface plasmon resonance biosensors.** An increase in resonance units indicates binding in real time of an injected analyte to a ligand on the sensor chip. *A*, interaction of HtpG (analyte) with immobilized DnaJ2 (ligand). *B*, interaction of DnaK2 with immobilized DnaJ2. *C*, interaction of HtpG with immobilized DnaK2.

TABLE 1

Constants obtained by SPR method

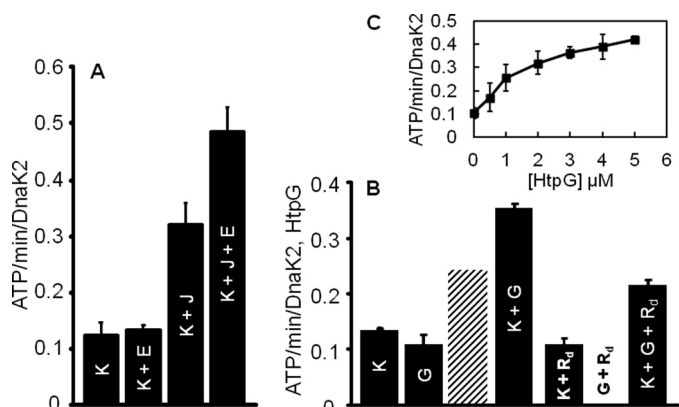| Complex	Association constant (k_a) (1/ms)	Dissociation constant (k_d) (1/s)	Equilibrium dissociation constant (K_D) (M)
DnaK2-DnaJ2	1.14×10^4	2.44×10^{-3}	1.73×10^{-7}
HtpG-DnaJ2	1.91×10^4	1.96×10^{-3}	1.02×10^{-7}
DnaK2-HtpG	3.22×10^3	8.29×10^{-3}	2.58×10^{-6}

FIGURE 4. **Enhancement of the ATPase activity of DnaK2 by co-chaperones and HtpG.** Data from three replicates are presented as mean ± S.E. *A*, effect of DnaJ2, and GrpE on the ATPase activity of DnaK2. In 1 ml of a reaction mixture, 4 nmol of DnaK2 (*K*), 0.8 nmol of DnaJ2 (*J*), and/or 0.4 nmol of GrpE (*E*) were present. *B*, effect of HtpG and radicicol on the ATPase activities of DnaK2 and HtpG. In 1 ml of a reaction mixture, 2 nmol of DnaK2 (*K*), 2 nmol of HtpG (*G*), and/or 3 nmol of radicicol (*R_d*) were present. The *hatched column* shows the activity that was obtained by adding the DnaK2 and HtpG activities measured separately. *C*, enhancement of the ATPase activity of DnaK2 by HtpG. The ATPase activity of DnaK2 was measured in 1 ml of a reaction mixture containing 2 nmol of DnaK2, 18 nmol of radicicol, and varying amounts of HtpG.

HtpG with radicicol whether ATP is essential for the enhancement of the DnaK2-assisted refolding by HtpG. The collaboration of the *E. coli* HtpG with the DnaK chaperone system in the assistance of refolding of heat-denatured luciferase and G6PDH depends on ATP (20). Radicicol inhibited this enhancement by HtpG, indicating that the collaboration of HtpG with the DnaK2/DnaJ2/GrpE chaperone system depended on ATP.

DMSO, a solvent for radicicol, showed no significant effect on the enhancement.

LDH that had been incubated at 25 °C in the presence of 5 M urea was inactivated to less than 2% of the initial activity, but recovered more than 20% of the initial activity when the urea solution was diluted 75-fold with a solution containing the DnaK2/DnaJ2/GrpE chaperone system (Fig. 5*D*). HtpG added with the chaperone system increased the refolding rate. As shown in Fig. 5*D*, radicicol suppressed the enhancement, whereas DMSO did not.

In contrast to G6PDH, MDH that had been denatured at 45 °C without HtpG (but in the presence of BSA, a control protein) did not refold significantly at 25 °C in the presence of the DnaK2 chaperone system as shown in Fig. 6, *C* and *D* (results indicated by *BSA→KJE*). Under the present experimental conditions, the enzyme may form aggregates, whose sizes are too large for the DnaK2 chaperone system to disaggregate and assist the enzyme's refolding (28). Supporting this, when MDH was incubated at 45 °C, it formed aggregates, as evidenced by the increase in the apparent absorbance at 360 nm or the light scattering (turbidity) of the enzyme solution (Fig. 6*A*), and the MDH aggregates were large enough to be precipitated by centrifugation. Representative molecular chaperones, including small heat shock proteins and Hsp90s are able to bind denatured substrates to suppress their aggregation. As shown in Fig. 6*A*, HtpG also suppressed the MDH aggregation in an HtpG concentration-dependent manner. Stoichiometry showed that one HtpG dimer could completely suppress the aggregation of a single heat-denatured MDH monomer. The formation of the HtpG-non-native MDH complex was confirmed by immuno-
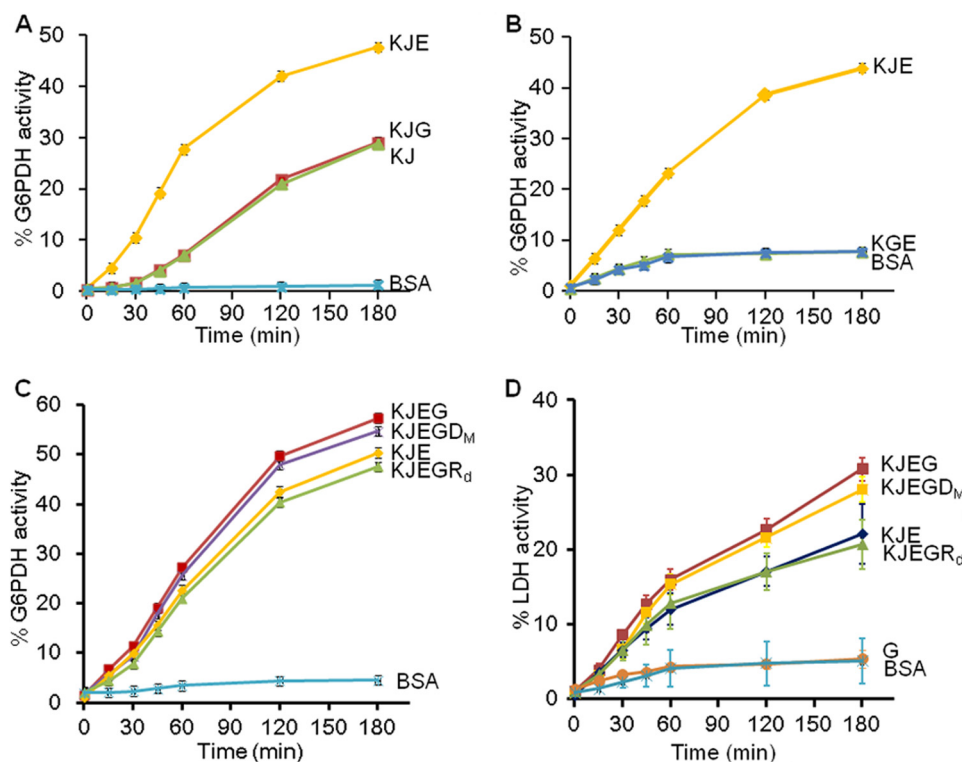
FIGURE 5. **Enhancement of the DnaK2-assisted protein refolding by DnaJ2, GrpE, and HtpG.** *A* and *B,* time-dependent reactivation of heat-denatured G6PDH (0.25 μM) in the presence of 6 μM DnaK2 (*K*), 1.2 μM DnaJ2 (*J*), 0.6 μM GrpE (*E*), and/or 0.5 μM HtpG (*G*). After heat treatment of G6PDH at 52 °C, the time course of changes in the enzyme activity was analyzed at 25 °C after addition of the chaperones and/or co-chaperones indicated in the figure. To measure a chaperone-independent folding, the same amount of BSA as the DnaK2 chaperone system (DnaK2/DnaJ2/GrpE) was added instead of the chaperone system. The same results as those in the presence of BSA were obtained when either only DnaK2 or DnaJ2 was added to the heat-denatured G6PDH. *C,* enhancement of the DnaK2 chaperone system-assisted G6PDH refolding by HtpG. The G6PDH refolding in the presence of the DnaK2/DnaJ2/GrpE chaperone system was carried out at 25 °C as described above. 0.5 μM HtpG (*G*) was added in the absence or in the presence of 1 μM radicicol (R_d) dissolved in dimethyl sulfoxide or the same volume of dimethyl sulfoxide (D_M) as that of the radicicol solution added. *D,* enhancement of the DnaK2 chaperone system-assisted LDH refolding by HtpG. After denaturation of LDH in the presence of urea and DTT at 25 °C, the time course of changes in the LDH (0.25 μM) activity was analyzed at 25 °C after addition of 6 μM DnaK2 (*K*), 1.2 μM DnaJ2 (*J*), 0.6 μM GrpE (*E*). 0.5 μM HtpG (*G*) was added in the absence or in the presence of radicicol (R_d) or dimethyl sulfoxide (D_M) as described above. Note that 0.5 μM HtpG (*G*) in the absence of the DnaK2 chaperone system did not enhance the refolding. Data from three replicates are presented as mean $\pm$ S.E. Some *error bars* are covered by plot symbols.

precipitation that showed a co-precipitation of the heat-denatured MDH with HtpG (Fig. 6*B*).

It has been shown that denatured MDH bound to the small Hsp IbpB is specifically delivered to the *E. coli* DnaK chaperone system, and then the enzyme refolds (29). This indicates that the small Hsp not only can bind denatured MDH to suppress its aggregation but also can hold it in a DnaK-dependent unfolding/folding-competent state. To elucidate the cooperative action of HtpG with the DnaK2 chaperone system for the enzyme's refolding, we tested whether the denatured MDH pre-bound to HtpG can be transferred to the DnaK2 chaperone system where it can be unfolded/refolded. When the DnaK2 chaperone system was added to the (heat-denatured) MDH-HtpG complex and the refolding reaction was carried out at 25 °C, MDH refolded well, as evidenced by the increase in the enzyme activity (Fig. 6*C*, results indicated by *HtpG→KJE*). In the absence of the DnaK2 chaperone system, no refolding took place (Fig. 6*C*, *HtpG→BSA*), indicating that HtpG by itself did not assist with the refolding of the enzyme and kept holding the denatured substrate. Therefore, the MDH refolding indicates that the bound substrate was transferred to DnaK2 from HtpG. We evaluated whether the ATP binding and its hydrolysis are necessary for the transfer from HtpG to DnaK2. To this end, we constructed mutants of HtpG that are defective in ATP hydro-

lysis. In *Saccharomyces cerevisiae* Hsp90 (Hsp82), Glu to Ala and Asp to Asn substitutions in the ATP-binding site at amino acid positions 33 and 79, respectively, abolish the Hsp82 ATPase activity (30). E33A can bind ATP but is incapable of its hydrolysis, whereas D79N cannot bind ATP. We constructed HtpG mutants HtpG(E34A) and HtpG(D79N), which are amino acid substitutions in the homologous residues. The ATPase activities of HtpG(E34A) and HtpG(D79N) were 16.3 $\pm$ 3.4% of the wild-type HtpG and nondetectable, respectively. The mutant was able to bind MDH and transfer it to the DnaK2 chaperone system where it refolded as shown in Fig. 6*C* (results indicated by *HtpG(E34A)→KJE* and *HtpG(D79N)→KJE*). Finally, we tested whether radicicol inhibits the transfer. As shown in Fig. 6*D* (*HtpG* + R_d→*KJE* and *HtpG* + D_M→*KJE*), the refolding took place regardless of the presence of radicicol or DMSO. Thus, ATP binding and its hydrolysis were not necessary for the transfer.

DISCUSSION

In this report, we showed that HtpG interacts specifically with DnaJ2 and DnaK2 in a yeast cell (Fig. 1). It interacted with them *in vitro* (Figs. 2 and 3). This is the first report that HtpG/Hsp90 interacts with DnaJ/Hsp40 or J-protein directly. The *E. coli* HtpG also interacts with DnaK to form a complex (20),
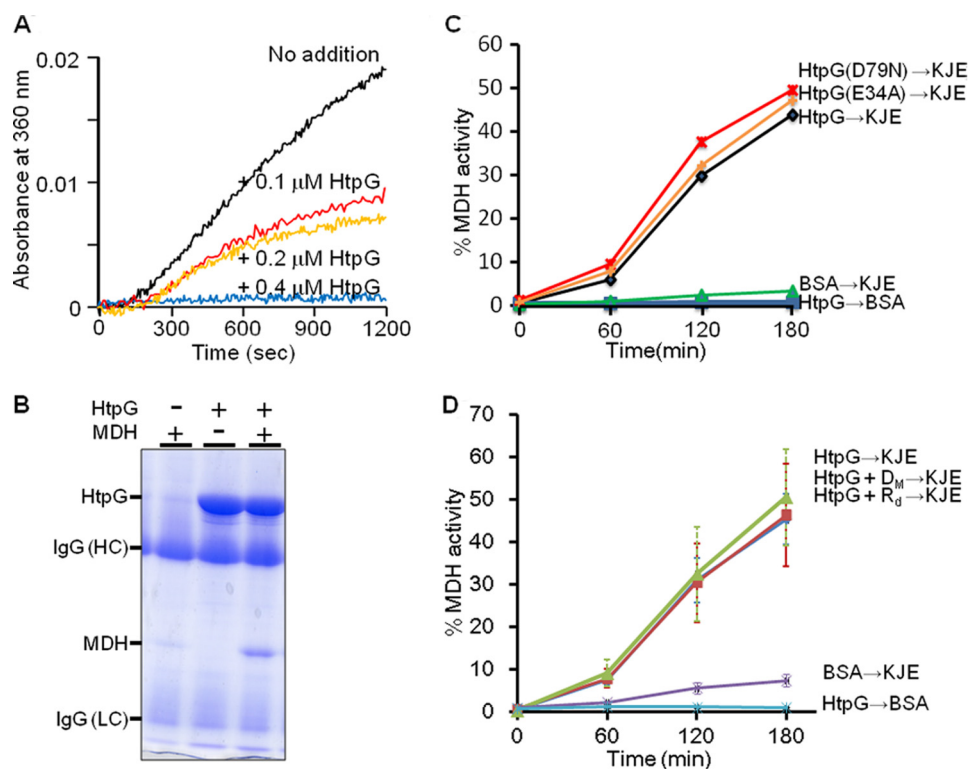
FIGURE 6. **Stable binding of the heat-denatured MDH to HtpG and refolding of the MDH by cooperative action between HtpG and the DnaK2 chaperone system.** *A*, suppression of aggregation of MDH by HtpG. 0.2 μM MDH was incubated in the absence (*No addition*) or presence of 0.1, 0.2, or 0.4 μM HtpG at 45 °C for 20 min. The apparent absorbance increase at 360 nm due to the aggregation of the heat-denatured MDH was measured. The absorbance changes in the presence of 0.2 μM HtpG without MDH were overlapped with those in the presence of 0.2 μM MDH and 0.4 μM HtpG and thus were removed from the figure for clarity. *B*, physical interaction of HtpG with MDH. For immunoprecipitation assays, a mixture containing 0.5 nmol of MDH and/or 1.0 nmol of HtpG was incubated at 45 °C for 25 min. After centrifugation, protein G-Sepharose 4 Fast Flow beads with coupled HtpG antibodies were added to the supernatant fraction and incubated for 2 h at 4 °C. Proteins co-precipitated with the beads were separated by SDS-PAGE (12%) and stained with Coomassie Brilliant Blue. *HtpG*, heavy chain of IgG (*IgG* (*HC*)), MDH, and light chain of IgG (*IgG* (*LC*)) are indicated. *C*, time-dependent refolding of MDH (0.25 μM) that had been denatured in the presence of a 2-fold greater concentration of HtpG, HtpG (D79N), or HtpG (E34A) at 45 °C for 30 min was followed at 25 °C in the presence of 5 μM DnaK2 (*K*), 10 μM DnaJ2 (*J*), and 5 μM GrpE (*E*). The *line* indicated by *HtpG→BSA* showed the results where MDH was heat-treated in the presence of 0.5 μM HtpG, and the refolding reaction at 25 °C was then carried out in the presence of BSA without the DnaK2 chaperone system. Note that 0.5 μM HtpG by itself did not enhance the refolding. Also note that the DnaK2 chaperone system could not assist the MDH refolding significantly when MDH was heat-denatured in the presence of BSA but in the absence of HtpG (*BSA→KJE*). *D*, time-dependent refolding of MDH (0.25 μM) that had been denatured in the presence of a 2-fold greater concentration of HtpG at 45 °C for 30 min was followed at 25 °C in the presence of 5 μM DnaK2 (*K*), 10 μM DnaJ2 (*J*), and 5 μM GrpE (*E*). 5 μM radicicol (*R_d*) or the same volume of dimethyl sulfoxide (*D_M*) as the radicicol was added. *C*, data from two replicates are presented as mean. *D*, data from three replicates are presented as mean ± S.E. Some *error bars* are covered by plot symbols.

although its affinity to DnaK has not been quantified. We determined the dissociation constants for the HtpG-DnaJ2, DnaK2-DnaJ2, and HtpG-DnaK2 complexes. To our surprise, DnaJ2 interacted with HtpG as strongly as DnaK2. However, HtpG interacted with DnaK2 much more weakly than DnaJ2. Based on these results, we hypothesize that DnaJ2 may be a scaffold protein that organizes the HtpG-DnaK2 chaperone complex. Our yeast two-hybrid experiments showed that a region of the DnaJ2 that is not the N-terminal 8-kDa J-domain, interacts with HtpG. Thus, HtpG may be able to bind DnaJ2 without interfering with the transient association of DnaJ2 with DnaK2, because DnaJ/Hsp40 interacts with DnaK/Hsp70 through the J-domain to enhance the ATP hydrolysis and the refolding of a substrate by DnaK (25). DnaJ2 may facilitate the interaction between HtpG and DnaK2 for the transfer of a substrate protein from DnaK2 to HtpG and vice versa. In eukaryotes, Hsp90 interacts with Hsp70 where Hop/Sti1 is an adaptor protein that directly associates with both Hsp70 and Hsp90 (31). Hsp90 influences the refolding activity of the Ssa1-Ydj1 complex in yeast only when Sti1 is present (32). There are no homologs of Hop/Sti1 in bacteria. Like Hop/Sti1, DnaJ2 may become a scaf-

fold protein for the interaction of HtpG with DnaK2 and play a role in the stimulation of the DnaK2-assisted refolding by HtpG. The physical interaction of DnaJ2 with HtpG and/or DnaK2 is different from that of Hop/Sti1 with Hsp90 and Hsp70 because DnaJ2 lacks the tetratricopeptide repeat domains, and both HtpG and DnaK2 do not have the (M/I)EEVD motif at their extreme C termini.

DnaJ/Hsp40 homologs or J-proteins can be divided into three subgroups based upon the degree of domain conservation (25). DnaJ2 belongs to type II J-proteins that have the G/F-rich domain but lack the cysteine-rich repeat or the so-called zinc finger-like domain. In fact, there is only one cysteine in DnaJ2, and the type I J-protein DnaJ1 conserves eight cysteines that form the domain. As shown in Fig. 7*A*, the cyanobacterial DnaJ2 is homologous to the *E. coli* type II J-protein CbpA. It has been shown that CbpA is particularly effective in making DnaK function with HtpG (20). Thus, the type II J-protein may play a special role for the cooperative action of HtpG with DnaK. However, we have to point out that the two J-proteins are different from each other in terms of their affinity toward a denatured substrate. CbpA can bind a substrate to prevent its aggre-
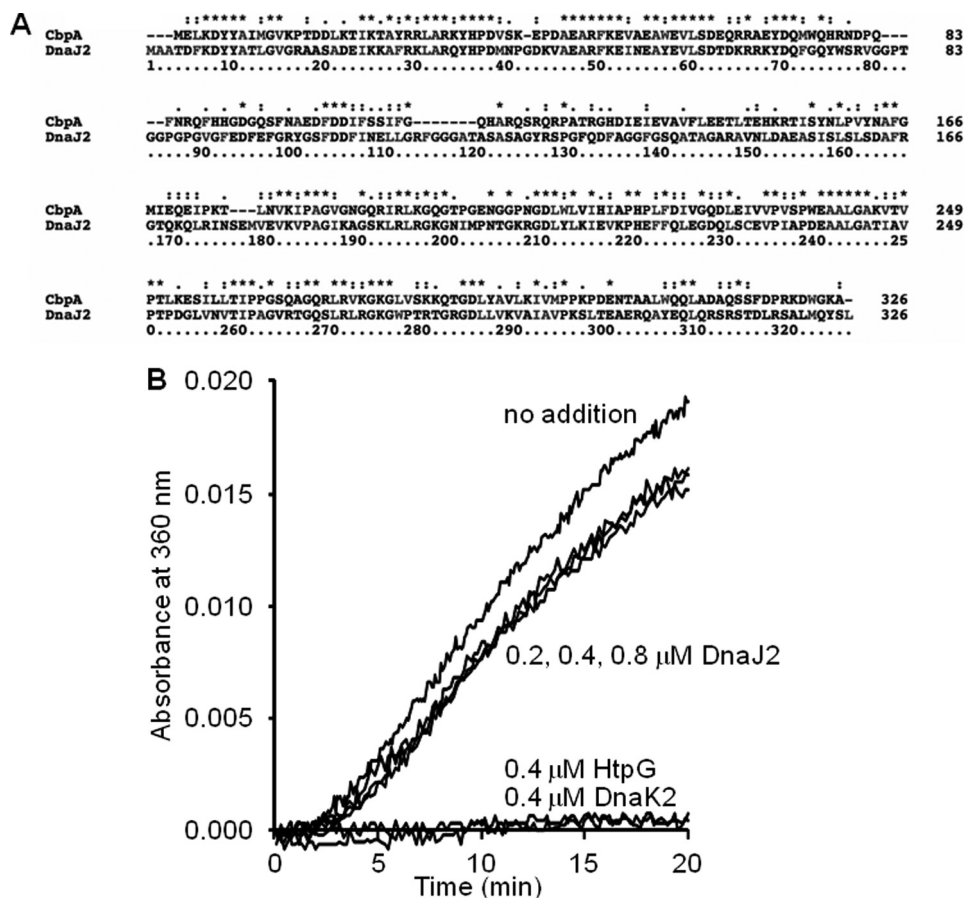
FIGURE 7. **Primary structures of type II J-proteins from *E. coli* and *S. elongatus* and the anti-aggregation activity of the *S. elongatus* DnaJ2 as compared with DnaK2 and HtpG.** *A,* sequence alignment for the *E. coli* CbpA and the *S. elongatus* DnaJ2. The sequences were aligned using Clustal X. *Dashes* represent gaps. An *asterisk* above the sequence indicates a position that has a single, fully conserved residue. *:* indicates that one of the following strong groups is fully conserved: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, or FYW. *.* indicates that one of the following weaker groups is fully conserved: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, or HFY. *B,* effect of DnaJ2, HtpG, or DnaK2 on the aggregation of MDH. 0.2 μM MDH was incubated in the absence (*No addition*) or presence of DnaJ2, HtpG, or DnaK2 at 45 °C for 20 min. The final concentrations of each chaperone and co-chaperone are indicated in the figure. The apparent absorbance increase at 360 nm was measured after addition of MDH.

gation (33), although DnaJ2 does not interact stably with the heat-denatured MDH because it could not suppress the enzyme's aggregation efficiently as compared with HtpG and DnaK2 (Fig. 7*B*). DnaJ2 did not suppress aggregation of heat-denatured citrate synthase either (data not shown).

Physical interaction of HtpG with DnaK2 resulted in the enhancement of the ATPase activity of DnaK2 like DnaJ2 (Fig. 4). However, HtpG did not substitute for DnaJ2 in the essential role of the DnaK2-assisted refolding of a denatured substrate (Fig. 5*B*). Furthermore, HtpG could not enhance the DnaK2-assisted refolding as a substitute for GrpE (Fig. 5*A*). However, HtpG enhanced the DnaK2-assisted refolding in the presence of DnaJ2 and GrpE (Fig. 5, *C* and *D*). These results indicate that each member has a distinct role in the HtpG/DnaK2/DnaJ2/GrpE chaperone system.

A denatured polypeptide that binds to DnaK/Hsp70 is known to stimulate the chaperone's ATPase activity (34). Thus, the enhancement of the ATPase activity of DnaK by HtpG may indicate that HtpG binds at or near the polypeptide-binding site of DnaK2, thereby promoting polypeptide release from DnaK2, while stimulating its transfer to HtpG. Radicicol inhibited the stimulation of the substrate refolding by HtpG (Fig. 5, *C* and *D*) because HtpG assisted the folding of a substrate transferred

from DnaK2 in an ATP-dependent manner, as documented recently for *E. coli* HtpG (20). The substrate transfer between the two prokaryotic chaperones is similar to that between eukaryotic chaperones (Fig. 8*A*). Some substrate polypeptides may not refold easily with the assistance of DnaK2 alone and thus will continue to associate with DnaK2. HtpG may forcefully release it from DnaK2 and thus help its refolding (Fig. 8*B-1*) in agreement with our finding that HtpG can enhance DnaK2-assisted refolding of a denatured polypeptide substrate.

In contrast to the urea-denatured LDH and heat-denatured G6PDH, heat-denatured MDH did not refold in the presence of the DnaK2 chaperone system as reported previously with the *E. coli* DnaK chaperone system (29). It was most likely due to the formation of large aggregates of denatured MDH, as described above. When heat-denatured in the presence of HtpG, the formation of aggregates was suppressed (Fig. 6*A*). However, the MDH appeared to be bound too strongly to be released from HtpG as no refolding was detected in the presence of ATP, unless the DnaK2 chaperone system able to scavenge misfolded species strongly associated with other chaperones (29) was present (Fig. 6*C*). In the substrate transfer from HtpG to DnaK2, ATP binding and its hydrolysis by HtpG was not involved, because mutations in the ATP binding and its
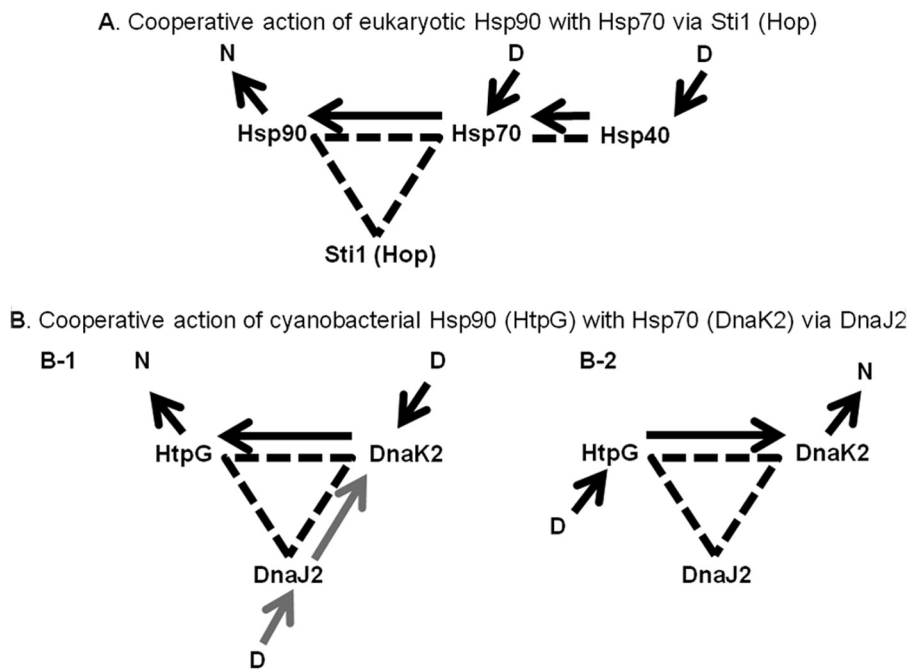
FIGURE 8. **Possible folding pathways for a denatured protein in the HtpG (Hsp90)/DnaK (Hsp70) chaperone system.** The *arrows* indicate the direction of a substrate transfer between chaperones/co-chaperone. *Broken lines* indicate the physical interaction between chaperones and co-chaperones. *D* and *N* indicate an unfolded/misfolded/denatured protein substrate and its native form, respectively. *A,* cooperative action between Hsp70 and Hsp90 in the eukaryotic system (32). A substrate is recruited by Hsp40 or Hsp70. The final assistance for the refolding is provided by Hsp90 after its receiving the substrate from Hsp70. This step is ATP-dependent. Sti1 or Hop interacts with both Hsp90 and Hsp70 physically to promote the chaperone's interaction and their chaperone function. *B,* cooperative action of cyanobacterial HtpG with DnaK2 via DnaJ2. We assume that DnaJ2 promotes the physical interaction between HtpG and DnaK2. *B-1,* in the case of urea-denatured LDH and heat-denatured G6PDH that do not form large aggregates, a protein substrate may be recruited by DnaJ2 and transferred to DnaK2. Alternatively, a substrate may bind to DnaK2 directly. Then, the refolding of the substrate may be completed by the assistance of DnaK2. If the substrate does not refold easily and repeats the binding and dissociation to/from DnaK2, it is transferred to HtpG to refold just like the eukaryotic system. This final step assisted by HtpG is ATP-dependent. HtpG enhances the ATPase activity of DnaK2, which may facilitate the cooperative action of DnaK2 and HtpG. *B-2,* in the case of an aggregation-prone protein like MDH, HtpG binds it to keep it soluble under stress, and then it is transferred to DnaK2 that assists its refolding under nonstress conditions. This substrate transfer between HtpG and DnaK2 is not dependent on ATP.

hydrolysis and the inhibitor radicicol did not prevent the transfer. Similar to the *E. coli* DnaK that assists the refolding of a substrate bound to the small Hsp IbpB that does not have the ATPase activity (29), DnaK2 translocates the substrate from HtpG to itself to assist with the refolding (Fig. 8*B-2*). Suggesting a possible more specific cooperative action between HtpG and the DnaK/DnaJ/GrpE chaperone system than that between IbpB and the DnaK/DnaJ/GrpE chaperone system, we found that as little as 0.5 μM HtpG was as effective as 4 μM IbpB (29) for binding heat-aggregating MDH to be subsequently handled by the DnaK chaperone system, and both conditions specifically produced the same net amount of natively refolded MDH (50 nM) in 100 min. Thus, together with the data of Veinger *et al.* (29), our *in vitro* experiments with heat-aggregated MDH suggest the existence of a functional overlap between the small heat shock proteins and HtpG, with regard to the ability to optimally present misfolded species to the DnaK unfolding system. Justifying the ratios between chaperones used here, recent proteomic studies show that HtpG is 5.8-fold less abundant than DnaK in the cytosol of *E. coli* cells (35).

It is not known how HtpG plays a role under stress. This study indicates that HtpG works closely with the DnaK2 chaperone system to take care of proteins that are damaged during stress. In the case of some proteins that tend to form large aggregates, HtpG may bind to form soluble complexes. These substrates can be then transferred to the DnaK2 unfolding system where following release they may refold to the native state

(Fig. 8*B-2*). Among proteins that do not form large aggregates, there are some that do not fold easily, even while being assisted by the DnaK2 chaperone system, in which case, the substrate may be transferred to HtpG, where it may refold with the assistance of the possible ATP-fueled unfolding activity of HtpG (Fig. 8*B-1*).

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