The Hsc66-Hsc20 Chaperone System in *Escherichia coli*: Chaperone Activity and Interactions with the DnaK-DnaJ-GrpE System

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Hsc66, a stress-70 protein, and Hsc20, a J-type accessory protein, comprise a newly described Hsp70-type chaperone system in addition to DnaK-DnaJ-GrpE in *Escherichia coli*. Because endogenous substrates for the Hsc66-Hsc20 system have not yet been identified, we investigated chaperone-like activities of Hsc66 and Hsc20 by their ability to suppress aggregation of denatured model substrate proteins, such as rhodanese, citrate synthase, and luciferase. Hsc66 suppressed aggregation of rhodanese and citrate synthase, and ATP caused effects consistent with complex destabilization typical of other Hsp70-type chaperones. Differences in the activities of Hsc66 and DnaK, however, suggest that these chaperones have dissimilar substrate specificity profiles. Hsc20, unlike DnaJ, did not exhibit intrinsic chaperone activity and appears to function solely as a regulatory co-chaperone protein for Hsc66. Possible interactions between the Hsc66-Hsc20 and DnaK-DnaJ-GrpE chaperone systems were also investigated by measuring the effects of co-chaperone proteins on Hsp70 ATPase activities. The nucleotide exchange factor GrpE did not stimulate the ATPase activity of Hsc66 and thus appears to function specifically with DnaK. Cross-stimulation by the co-chaperones Hsc20 and DnaJ was observed, but the requirement for supraphysiological concentrations makes it unlikely that these interactions occur significantly in vivo. Together these results suggest that Hsc66-Hsc20 and DnaK-DnaJ-GrpE comprise separate molecular chaperone systems with distinct, nonoverlapping cellular functions.

The Hsp70, or stress-70, protein family is a ubiquitous class of proteins of ~70 kDa, which function as ATP-dependent molecular chaperones (reviewed in references 5, 16, 17, 20, 21, 34, and 36). Hsp70 proteins have been shown to play roles in de novo protein folding, degradation of misfolded proteins, membrane trafficking, regulatory processes, and maintaining cell viability upon stress. A number of different Hsp70 isoforms have been identified in eukaryotes, but only a single Hsp70, DnaK, has been characterized in prokaryotes. Recently, a second prokaryotic family member, encoded by the *DnaK* gene and designated Hsc66 (for heat shock cognate *M* of ~66 kDa), was identified in *Escherichia coli* (26, 54). DNA sequence data from a number of other bacteria, including *Actinobacillus actinomycetemcomitans* (47), *Azotobacter vinelandii* (68), *Buchnera aphidicola* (8), *Haemophilus influenzae* (14), *Neisseria gonorrhoeae* (48), *Neisseria meningitidis* (39), *Pseudomonas aeruginosa* (44), and *Salmonella typhimurium* (63) indicate that Hsc66 in addition to DnaK occurs widely.

The cellular function of Hsc66 has not been determined. In *E. coli*, Hsc66 is constitutively expressed at a level similar to that of DnaK, comprising ~1% of the total cellular protein, but unlike DnaK, Hsc66 levels do not increase significantly upon heat shock (62). The high constitutive expression of Hsc66 and lack of induction by thermal stress suggest an important cellular role under normal growth conditions. Disruption of the *hscA* gene in *E. coli*, however, does not result in any gross phenotypic changes (26, 63) and has not as yet provided insight into the function of Hsc66. In contrast, *dnaK* null mutants have major growth defects (6, 41), suggesting that Hsc66 has function(s) separate from those of DnaK. ATPase activity consistent with its role as an ATP hydrolysis-coupled chaperone has been demonstrated for Hsc66 (62), but chaperone-like activities (prevention of protein aggregation and assisted protein folding) and coupling of ATP binding and hydrolysis with polypeptide binding affinity have not been shown.

The chaperone activities of DnaK and other Hsp70 chaperones are regulated by DnaJ and Hsp40 accessory proteins (~40 kDa) which stimulate the ATPase activity of the chaperone (31), and this interaction is mediated by an N-terminal J-domain segment (27, 64). The ATPase activity of Hsc66 is regulated by Hsc20 (62), a 20-kDa protein encoded by the *hscB* gene (27). The N-terminal 70-residue sequence of Hsc20 exhibits similarities to the N-terminal J-domain sequence of DnaJ and Hsp40 proteins, including the His-Pro-Asp J-motif signature sequence (3) and hydrophobic core residues observed in J-domain nuclear magnetic resonance structures (43, 45, 59). The remainder of Hsc20 (residues 71 to 171), on the other hand, is not homologous to the C-terminal region of DnaJ or other Hsp40 proteins and lacks the Gly- and Phe-rich, Cys-rich zinc finger, and C-terminal segments shown to be important for both Hsp70 interactions and J-protein chaperone activity (57, 64). Homologs of the *hscB* (*Hsc20*) gene are also found adjacent to the *hscA* (*Hsc66*) gene in each of the organisms listed above. Hsc20 thus appears to represent a new subfamily of J-type co-chaperones. These “small Jac’s” (for J-type accessory chaperones) (~20 kDa) each contain a N-terminal J-domain presumed to mediate interactions with Hsc66 and a unique C-terminal domain whose function is unknown. The similarity of the J-domain of Hsc20 to that of DnaJ raises the question of whether “cross-talk” between the two chaperone systems might occur, i.e., interaction of Hsc20 with DnaK as well as with Hsc66 and interaction of DnaJ with Hsc66 as well as with DnaK. DnaK is additionally subject to regulation by GrpE, which facilitates exchange between ADP and ATP (31), but possible interactions between GrpE and Hsc66 have not been investigated.

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To investigate the chaperone activity of Hsc66, we have studied its ability to prevent aggregation of three model sub-structate proteins (rhodanese, citrate synthase, and luciferase) as well as nucleotide effects on this activity. We have also investigated possible interactions between the Hsc66:Hsc20 and DnaK-DnaJ-GrpE chaperone systems by measuring cross-stimulation of chaperone ATPase activities.

**MATERIALS AND METHODS**

**Materials.** The DnaK expression plasmid pM2 was provided by G. C. Walker. E. coli W3110 was from the American Type Culture Collection (ATCC 27232). DH5α F- IQ cells were from Gibco-BRL, and BL21 (DE3) pLysS cells were from Novagen. Enzymes for DNA manipulation were obtained from Boehringer-Mannheim Corp., New England Biolabs, Inc., or U.S. Biochemical Corp. Synthetic oligonucleotides were obtained from Operon Technologies. Bacterial growth medium components were from Difco, and other reagents were from Sigma Chemical Co.

**Overexpression and purification of Hsc66, Hsc20, DnaK, DnaJ, and GrpE.** Hsc66, Hsc20, DnaK, and DnaJ were expressed and purified as previously described (62). The DnaJ and GrpE expression vectors, pTrcDnaJ and pTrcGrpE, respectively, were constructed by PCR amplification of their genes from genomic DNA isolated from E. coli K-12 strain W3110 and cloning them into pTrc99a (Pharmacia).

BL21 cells transformed with pTrcDnaJ were grown in Terrific broth (51) at 37°C. Protein expression was induced with 0.5 mM isoproprilβ-D-galactoside (IPTG) at an A600 of ~1. After ~16 h, cells were harvested by centrifugation, frozen, thawed, and lysed in a French pressure cell in a solution containing 50 mM HEPES (pH 7.5), 0.5 mM EDTA, 1 mM dithiothreitol (DTT) with 0.1% Triton X-100, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF) to inhibit proteolysis. The supernatant fluid following centrifugation at 20,000 × g for 30 min was combined and diluted with an equal volume of a solution containing 100 mM HEPES (pH 6.5), 1 mM DTT, and 0.5 mM EDTA. This solution was passed over a DEAE-cellulose column (DE-52; Whatman), and the unbound material was passed over a DEAE-cellulose column on a fast protein liquid chromatography system (Pharmacia) by the method of Schonfeld et al. (52) to purify Hsc66 and to refold it into a solution containing assay buffer, nucleotides, and indicated amounts of chaperones at 30°C. The mixture was incubated at 25°C for 1 h. Aggregation was performed by diluting refolded luciferase 100-fold into a solution containing assay buffer and indicated amounts of chaperones. Aggregation was measured by monitoring changes in turbidity at 320 nm. Assays were carried out in a 1-ml cuvette with a 1-cm path length without stirring for 30 min.

**Luciferase aggregation assays.** Luciferase (Sigma Chemical Co.) was stored as a 158 μM (8.2-μg/ml) crystalline suspension at 4°C. Thermal aggregation was performed by incubating a solution containing assay buffer, nucleotides, and indicated amounts of chaperones at 30°C for 10 min. Crystalline citrate synthase was diluted 100-fold into this mixture and mixed rapidly for 15 s, and aggregation was monitored by measured changes at 320 nm as described by Lee (29). Assays were carried out at 4°C in a 1-ml cuvette with a 1-cm path length without stirring for 30 min.

**RESULTS**

**Effects on protein aggregation.** Because no endogenous substrate proteins have been identified for Hsc66, we investigated its chaperone activity by the ability to suppress aggregation of denatured bovine rhodanese, porcine citrate synthase, and firefly luciferase. These proteins have been used previously as model substrates for other chaperones, including members of the Hsp25 (13, 24), Hsp40 (10, 12, 32, 57) Hsp70 (30, 37, 53, 64), Hsp90 (4, 25, 67) families. For studies utilizing rhodanese and luciferase, proteins were denatured using 6 M guanidine hydrochloride prior to incubation with Hsc66. Aggregation was initiated by diluting this solution into a reaction mixture containing Hsc66 at 25°C and monitored by measuring increases in absorbance due to changes in turbidity. In the case of citrate synthase, the native protein was diluted into a reaction mixture containing Hsc66 at 43°C and aggregation was monitored by measuring absorbance increases.

The effects of various concentrations of Hsc66 on the aggregation of denatured rhodanese are shown in Fig. 1A. Partial suppression of aggregation was observed at a molar ratio of Hsc66 to rhodanese of 2:1, but complete (>90%) suppression

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over the time course of the experiment required ratios of >8:1. The high concentration of Hsc66 relative to that of rhodanese required for complete protection is similar to that observed for other chaperones (67) and likely reflects both the affinity of the chaperone for the unfolded polypeptide and the presence of multiple binding sites on the unfolded protein. The effects of nucleotides on the activity of Hsc66 are shown in Fig. 1B. Under the conditions used, ADP has little or no effect on activity, whereas ATP reduces the ability of Hsc66 to suppress aggregation by about half. These findings are consistent with models for Hsp70 regulation in which ADP stabilizes a chaperone conformation having high substrate affinity, thereby affording protection from aggregation, whereas ATP favors a more rapidly exchanging state which allows for refolding and/or aggregation (35, 42, 58). Thus, the cellular ratio of ADP to ATP and their exchange rates will determine the activity of Hsc66. For comparison, a similar experiment on the effect of DnaK on rhodanese aggregation is shown in Fig. 1C. At the concentration shown, DnaK was somewhat less effective than Hsc66 in suppressing aggregation, although complete suppression was obtained with molar ratios of DnaK to rhodanese greater than 10:1 (data not shown). Nucleotides have similar effects on DnaK activity as for Hsc66, with ATP reducing the aggregation suppression and ADP having little effect.

The effect of Hsc66 on the aggregation of citrate synthase is shown in Fig. 2. Figure 2A shows that aggregation is effectively suppressed at a ratio of Hsc66 to citrate synthase of >3:1. The lower molar ratio of Hsc66 required for suppression of aggre-
actions with unfolded proteins (32), and Hsc20 lacks a similar region. Possible effects of Hsc20 on Hsc66 chaperone activity in the rhodanese and citrate synthase aggregation suppression assays were also studied. No effect of Hsc20 was observed in the absence of nucleotide or in the presence of ADP or 400 μM ATP (data not shown). At low levels of ATP (<80 μM), Hsc20 enhanced the activity of Hsc66. This effect, however, appears to result from stimulation of the ATPase activity of Hsc66, rather than a direct effect on peptide binding. At ATP concentrations of <80 μM, ATP is depleted during the course of the assay, and the resulting ADP complex is expected to exhibit greater aggregation suppression. Thus, Hsc20 appears to stimulate chaperone activity solely by elevating the ATPase activity of Hsc66. This can be contrasted with DnaJ, which not only stimulates the ATPase activity of DnaK but also acts to target peptide substrates to the chaperone (58).

**ATPase activities of the Hsc66 and DnaK chaperone systems.** Because the chaperone activities of Hsp70 proteins are regulated by nucleotides, it was of interest to compare the ATPase activities of the Hsc66-Hsc20 and DnaK-DnaJ-GrpE systems. For this purpose, assays were performed with chaperone and cochaperone concentrations approximating those which occur in vivo. The cellular concentrations of Hsc66 and Hsc20 are estimated to be ~20 and ~10 μM, respectively (62), and those of DnaK, DnaJ, and GrpE are ~20, ~1, and ~10 μM, respectively, under nonstress conditions (1, 23, 38). Table 1 shows turnover numbers (in moles of P_i produced per mole of chaperone per minute) for Hsc66 and DnaK in the presence and absence of their respective cochaperones. Assays were performed with 400 μM ATP, which should yield maximal activities, assuming that Hsc66 has high affinity for ATP as found for DnaK (K_m of ~1 nM) (50). Under these conditions, the intrinsic ATPase activity of Hsc66 (in the absence of Hsc20) is approximately threefold higher than that for DnaK alone. In the presence of a physiological level of Hsc20, the activity of Hsc66 was stimulated ca. twofold, whereas a physiological level of DnaJ stimulated DnaK to a greater extent, ~5.5-fold. Because of the higher intrinsic ATPase activity of Hsc66, however, the Hsc66-Hsc20 and DnaK-DnaJ reaction mixtures exhibit similar total activities. Addition of GrpE, which catalyzes nucleotide exchange with DnaK, increased the overall ATPase rate of the DnaK-DnaJ-GrpE system to a value ca. twofold greater than that observed for DnaK alone. These results suggest that the Hsc66-Hsc20 and DnaK-DnaJ-GrpE systems have roughly similar ATPase activities, but it should be emphasized that actual physiological ATPase activities of the two chaperone systems will depend critically on the exact cellular concentrations of the chaperone proteins (see below) as well as any additional regulatory factors that remain to be identified.

We next investigated the possibility of interactions between

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**TABLE 1. ATPase activities of Hsc66-Hsc20 and DnaK-DnaJ-GrpE chaperone systems.**

<table>
<thead>
<tr>
<th>Chaperone(s)</th>
<th>Turnover (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 μM Hsc66</td>
<td>0.12</td>
</tr>
<tr>
<td>+ 10 μM Hsc20</td>
<td>0.24</td>
</tr>
<tr>
<td>20 μM DnaK</td>
<td>0.04</td>
</tr>
<tr>
<td>+ 1 μM DnaJ + 10 μM GrpE</td>
<td>0.22</td>
</tr>
<tr>
<td>+ 1 μM DnaJ + 10 μM GrpE</td>
<td>0.47</td>
</tr>
</tbody>
</table>

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*Assays were carried out using 400 μM ATP in 100 mM HEPES (pH 7.5)–150 mM KCl–24 mM NaCl–1 mM DTT; reaction mixtures including DnaJ additionally contained 0.02% Triton X-100.
the Hsc66-Hsc20 and DnaK-DnaJ-GrpE systems by assaying for cross-stimulation of the ATPase activity of Hsc66 and DnaK by Hsc20, DnaJ, and/or GrpE. Figure 4 compares the effects of Hsc20 and DnaJ on the ATPase activity of Hsc66. The results are plotted as the increase in ATPase activity relative to the activity in the absence of cochaperone. Assuming 1:1 stoichiometry, hyperbolic saturation curves were obtained when the data were corrected for bound cochaperone. Extrapolation to saturating levels of Hsc20 indicate a maximal stimulation of approximately sixfold, and the concentration of Hsc20 required for half-maximal stimulation (~12 μM) is in the same range as the estimated cellular concentration of the cochaperone. DnaJ stimulated Hsc66 to a similar maximal extent (ca. fivefold), but the concentration required for half-maximal stimulation was ~110 μM. This value is ~100-fold higher than the cellular level of DnaJ under nonstress conditions and ~10-fold greater than under heat shock conditions (1), suggesting that DnaJ is not likely to affect the activity of Hsc66 to a significant extent in vivo.

Figure 5 shows a comparison of the effects of Hsc20 and DnaJ on the ATPase activity of DnaK. Hsc20 stimulated DnaK only very weakly at the concentrations tested. Maximal stimulation was estimated to be ca. twofold with half-maximal stimulation requiring >200 μM Hsc20, a value ~20-fold higher than normal cellular levels of Hsc20 (62). DnaJ, in contrast, stimulated DnaK ATPase activity ~12-fold, and half-maximal stimulation occurred at ~1.8 μM DnaJ, near the cellular concentration for DnaJ (1). The high, nonphysiological concentrations of Hsc20 and DnaJ required for cross-stimulation of DnaK and Hsc66, respectively, suggest Hsc66 and Hsc20 comprise a chaperone pair that functions separately from DnaK and DnaJ in vivo.

GrpE, which functions as a nucleotide exchange factor and stimulates the ATPase activity of DnaK (31), was also assayed for effects on the ATPase activity of Hsc66 (Fig. 6). No stimulation was observed at GrpE concentrations of up to 30 μM either in the presence or absence of Hsc20. When assays were performed in the presence of high concentrations of DnaJ, however, a small degree of stimulation was observed, and the effect of GrpE on Hsc66 ATPase activity in the presence of 50 μM DnaJ is shown. Extrapolation to saturating concentrations of GrpE indicates a maximal stimulation of ~1.6-fold with half-maximal stimulation at ~5 μM GrpE. This effect can be contrasted with the effects of GrpE on the ATPase activity of DnaK (Fig. 6; see also reference 31). GrpE stimulates DnaK in

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**FIG. 4.** Effects of Hsc20 and DnaJ on ATPase activity of Hsc66. Increases in basal ATPase activity at 25°C are plotted as a function of free cochaperone concentration. The concentration of Hsc66 was 5 μM. The ATP concentration was 5 mM, and the buffer contained 0.1 M HEPES (pH 7.5), 0.1 M KCl, 24 mM NaCl, and 10 mM MgCl2. Concentrations of free cochaperone were calculated assuming 1:1 binding stoichiometry with Hsc66 by using the equation

\[
[\text{cochaperone}]_{\text{total}} - [\text{cochaperone}]_{\text{bound}} = -E \times \Delta V_{\text{max}}
\]

where \([\text{cochaperone}]_{\text{total}}\) is the concentration of added cochaperone, \(E\) is the observed rate change at \([\text{cochaperone}]_{\text{total}}\), \(V_{\text{max}}\) is the rate increase extrapolated to infinite cochaperone concentration. Curves represent calculated hyperbolic saturation functions, assuming maximal stimulation of 6.3-fold for Hsc20 and 12.2-fold maximal stimulation and half-maximal stimulation at 12 μM for Hsc20 and 100 μM for DnaJ. 

**FIG. 5.** Effects of Hsc20 and DnaJ on ATPase activity of DnaK. Increases in basal ATPase activity at 25°C are plotted as a function of total cochaperone concentration. The concentration of DnaK was 20 μM; other conditions are as given in the legend to Fig. 3. Curves represent hyperbolic saturation functions, assuming a maximal stimulation of 2.3-fold and half-maximal stimulation at 280 μM for Hsc20 and 12.2-fold maximal stimulation and half-maximal stimulation at 1.8 μM for DnaJ.

**FIG. 6.** Effects of GrpE concentration on the ATPase activity of Hsc66 and DnaK. Increases in basal ATPase activity at 25°C are plotted as a function of total cochaperone concentration. Five micromolar Hsc66 or 10 μM DnaK was used where indicated. Other conditions are as described in the legend to Fig. 3. The curves shown represent hyperbolic saturation functions, assuming a maximal stimulation of 1.6-fold for Hsc66 with DnaJ, 1.7-fold for DnaK in the absence of DnaJ, and 3.8-fold for DnaK with DnaJ, with half-maximal stimulation at GrpE concentrations of 5.0, 1.2, and 2.8 μM, respectively.
the absence of DnaJ (maximal stimulation ∼1.7-fold, half-
maximal stimulation ∼1.2 μM) and with physiological concen-
trations of DnaJ causes even greater stimulation (3.8-fold
above the DnaJ-stimulated activity and ∼53-fold above DnaK
alone). Previous studies have also shown that GrpE is able to
form a stable 2:1 complex with DnaK in the absence of nucle-
otides (52), and we used size exclusion chromatography to
investigate the possibility of interactions of GrpE with Hsc66
which may not be manifested as effects on ATPase activity.
No complex formation could be detected between Hsc66 and
GrpE, whereas a GrpE-DnaK complex was observable under
identical conditions (data not shown). These findings suggest
that while GrpE is able to stimulate the ATPase activity of
Hsc66 to a small degree in the presence of supraphysiological
levels of DnaJ, GrpE is not likely to function as a co-chaperone
with Hsc66 under normal cellular conditions.

DISCUSSION

The bacterial DnaK, DnaJ, and GrpE proteins comprise one
of the first chaperone systems described and remain the pro-
totypical Hsp70 model. It had been generally assumed that in
prokaryotes this single Hsp70 system was sufficient for cellular
functioning, and the discovery of genes encoding a second
Hsp70-type chaperone (hscA) and J-type co-chaperone (hscB)
in E. coli was surprising (26, 54). (Other genes encoding pro-
tective sequences similar to regions of DnaK and
Hsp70 proteins and DnaJ and Hsp40 proteins have subse-

dently been identified in E. coli [60].) The amino acid se-
quence of the hscA gene product, Hsc66, suggests that its
overall structure is similar to that of DnaK and other Hsp70
proteins, but the relatively low overall sequence identity
(~40%) suggests that important functional differences may
exist between the two chaperones. In addition, the hscB gene
product, Hsc20, differs significantly from DnaJ and other J-
type co-chaperones, exhibiting low (<15%) amino acid se-
quence identity in the N-terminal J-domain and having a short
C-terminal domain in place of other segments commonly
found in Hsp40 proteins. These differences between Hsc66
and Hsc20 compared to other Hsp70 systems raise questions
regarding the function and regulation of the Hsc66-Hsc20 sys-
tem.

In previous studies, we found that Hsc66 possesses a low
basal ATPase activity typical of Hsp70 proteins and that this
intrinsically active is stimulated by Hsc20 (62). The results de-
scribed herein show that when assayed using concentrations
approximating those found in vivo, the Hsc66-Hsc20 and
DnaK-DnaJ-GrpE systems exhibit roughly similar ATP hydro-
lisis activities, suggesting that the two systems may possess
similar chaperone capacities in vivo. Furthermore, studies on
suppression of aggregation of denatured model substrate pro-
teins establish that Hsc66 exhibits chaperone activity in a
nucleotide-dependent manner, as expected for an ATP hy-
drolysis-coupled system. The results are consistent with the
generally accepted model for Hsp70 action (42) in which ATP
destabilizes Hsc66-peptide complexes, and peptide binding
and release are coupled to ATP hydrolysis and ADP-ATP
exchange rates. Hsc20, on the other hand, does not exhibit
intrinsically chaperone activity and appears to act strictly as a
co-chaperone to regulate the ATPase activity of Hsc66. This
can be contrasted with the role of DnaJ, which can associate
with substrate proteins and exhibits intrinsic chaperone activity
in addition to its regulatory effects on DnaK ATPase activity
(22, 57). Hsc20 lacks domains commonly found in DnaJ and
other Hsp40 proteins, and the function of the small C-terminal
domain present in Hsc20 is not known.

Whereas none of the model substrate proteins tested are
found in E. coli, the different chaperone activities of Hsc66
and DnaK imply important differences between the two chaper-
ones. With rhodanese, both Hsc66 and DnaK were effective in
suppressing aggregation, although slightly lower molar ratios
of Hsc66 were required for complete protection. With citrate
synthase and luciferase, Hsc66 was effective in suppressing
aggregation only with the former, whereas DnaK was effective
only with the latter. These differences in substrate specificity
profiles presumably arise from structural differences in the
peptide binding domains of the two chaperones. Alignment of the
amino acid sequence of Hsc66 with the β-sandwich subdo-
main of DnaK shown to bind peptide (69) reveals that only
~50% of the residues are conserved, and 7 of 16 residues
directly contacting bound peptide in DnaK are replaced with
other amino acids in Hsc66. Peptide binding preferences have
not been established for Hsc66, but based on these sequence
dissimilarities, they are likely to differ from those determined
for DnaK (15, 49). In the case of citrate synthase, it is note-
worthy that Hsc66 is active in suppressing aggregation at tem-
peratures causing heat shock in E. coli (42°C). Our previous
studies demonstrated that Hsc66 ATPase activity increases
with temperature up to 50°C (62), and the chaperone and
ATPase activities of Hsc66 at elevated temperatures suggest
that Hsc66 maintains function under thermal stress as well as
under normal growth conditions.

Despite differences in substrate specificity profiles, the over-
all similarities between Hsc66 and DnaK and between Hsc20
and DnaJ raised the question of whether the proteins function
independently or whether heterologous interactions might re-
sult in “cross-talk” between the two systems. Based on the
results obtained here using ATPase stimulation as a measure
of cross-reactivity, the requirement for supraphysiological
co-chaperone concentrations for cross-stimulation make it appear
unlikely that these interactions occur to any significant extent
in vivo. The finding that DnaJ can stimulate Hsc66 ATPase
activity when present at sufficiently high concentrations to favor binding, however, suggests that some key structural features are shared by DnaJ and Hsc20. Figure 7A shows schematic representations of Hsc20 and DnaJ. Both proteins have N-terminal J-domains of ~70 residues, but DnaJ has a large C-terminal segment (~33 kDa) containing a glycine-rich linker region, a cysteine-rich zinc finger-like region, and a peptide binding region (7, 11, 46, 56), whereas Hsc20 has a smaller C-terminal domain (~12 kDa) that is predicted to fold as a coiled-coil structure (9). Studies with truncated forms of DnaJ have suggested that both the J-domain and glycine-rich linker region are required for interaction with DnaK (27, 64), and the lack of a glycine-rich linker region coupled with differences in the J-domain in Hsc20 may explain the very low activity of Hsc20 with DnaK. Other studies in which the glycine-rich region was deleted, however, have shown that this did not affect the ability of DnaJ to stimulate the ATPase activity of DnaK (65). This points to differences in J-domain sequences as the primary cause of the weak interaction of Hsc20 with DnaK. In contrast, DnaJ is able to substitute for Hsc20 in stimulating the ATPase activity of Hsc66, although higher concentrations are required. The activity of DnaJ with Hsc66 implies that the major interactions with Hsc66 are mediated by the J-domain common to both DnaJ and Hsc20 and that the C-terminal domain of Hsc20 is not essential for ATPase stimulation. An alignment of the amino acid sequences of the J-domain regions of Hsc20 and DnaJ provides insight into residues which might be critical for this interaction (Fig. 7B). Residues conserved in the two proteins include the J-motif signature sequence, HPD, and adjacent amino acids as well as several residues observed to be buried in the hydrophobic cores of the DnaJ (43, 60) and Hsp40 (45) J-domains. Only residues exposed on the surface would be available for binding to Hsc66, suggesting that the YHPDK sequence at positions 31 to 35 of Hsc20 is likely to be sufficient to specify the interaction. The corresponding sequence is observed to occur in a surface-exposed loop in nuclear magnetic resonance structures of fragments of DnaJ (43) and Hsp40 (45). Mutation of the histidine residue of the HPD sequence in DnaJ (64) and Ydj1 (61) has been shown to lead to inactivation, establishing the importance of this region, and we have found that a His32→Cys mutant of Hsc20 is also inactive (55).

The activity of the DnaK system is also subject to regulation by GrpE, which increases rates of peptide binding and release by facilitating exchange between ADP and ATP. Analysis of the complete sequence of the E. coli K-12 genome (2) reveals a single GrpE-type protein, suggesting the possibility that GrpE could function as a nucleotide exchange factor for Hsc66 in addition to DnaK. However, no interactions between GrpE and Hsc66 were detectable by using either ATPase stimulation or size exclusion chromatography. Analysis of the crystal structure of the DnaK-GrpE complex reveals a large number of contacts between the two proteins that span multiple regions of the ATPase domain (19). Alignment of the sequence of Hsc66 with DnaK reveals that only 4 of 21 amino acids in DnaK in contact with GrpE are identical, and while the relative importance of individual residues has not been determined, the numerous differences present in Hsc66 may preclude binding of GrpE. In this regard, Hsc66 behaves like eukaryotic cytosolic Hsp70 proteins which do not appear to utilize a GrpE-like cochaperone (37). In these cases, nucleotide exchange may not be a rate-limiting step in the chaperone cycle as it is for DnaK (50). The finding that GrpE stimulated Hsc66 ATPase activity in the presence of high levels of DnaJ is surprising and suggests that the possibility that GrpE and DnaJ may directly interact when modulating Hsp70 ATPase activity.

The specific cellular function(s) of Hsc66 and Hsc20 is not known, but their relatively high expression levels under non-stress conditions imply important general housekeeping roles. Some inferences regarding their function(s) can be drawn from analysis of recent genome sequence data. Sequences of the genomes of several bacteria, including E. coli (2), H. influenzae (14), B. aphidicoa (8), N. gonorrhoeae (48) and P. aeruginosa (44), reveal that each of these organisms has a gene cluster containing hscA and hscB homologs together with genes homologous to Fe-S cluster maturation genes (nif genes) of nitrogen-fixing bacteria. An analogous gene cluster separate from the nif genes was also recently identified in the nitrogen-fixing bacterium A. vinelandii (68). The occurrence of nif-like genes in non-nitrogen-fixing organisms and their counterparts in A. vinelandii led Zheng and coworkers to propose that these genes might play a role in formation or repair of iron sulfur proteins and to designate them as isc (iron sulfur cluster) genes (68). The sequential gene arrangement in the cluster, iscSU-hsbcBA-fdxs, is similar in each of the above organisms, and it appears likely that the genes are cotranscribed and encode proteins with coupled functions. Thus, the role of the Hsc66-Hsc20 chaperone system may be to function together with the iscS, iscU, iscA, and fdx genes to assist in protein folding steps involved in assembly and/or repair of iron sulfur clusters in Fe-S proteins.

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