Hsc66 and Hsc20, a new heat shock cognate molecular chaperone system from *Escherichia coli*

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Abstract

The *hscA* and *hscB* genes of *Escherichia coli* encode novel chaperone and co-chaperone proteins, designated Hsc66 and Hsc20, respectively. We have overproduced and purified Hsc66 and Hsc20 in high yield in *E. coli* and describe their initial characterization including absorbance, fluorescence, and circular dichroism spectra. Immunoblot analyses of *E. coli* cultures using antisera to Hsc66 and Hsc20 raised in rabbits establish that Hsc66 and Hsc20 are constitutively expressed at levels corresponding to cell concentrations $\sim 20 \mu\text{M}$ and $\sim 10 \mu\text{M}$, respectively. The levels do not change appreciably following heat shock (44°C), but a small increase in Hsc20 is observed following a shift to 10°C . Purified Hsc66 exhibits a low intrinsic ATPase activity ($\sim 0.6 \text{ min}^{-1}$ at 37°C), and Hsc20 was found to stimulate this activity up to 3.8-fold with half-maximal stimulation at a concentration $\sim 5 \mu\text{M}$. These findings suggest that Hsc66 and Hsc20 comprise a molecular chaperone system similar to the prokaryotic DnaK/DnaJ and eukaryotic hsp70/hsp40 systems. Sequence differences between Hsc66 and Hsc20 compared to other members of this chaperone family, however, suggest that the Hsc66/Hsc20 system will display different peptide binding specificity and that it is likely to be subject to different regulatory mechanisms. The high level of constitutive expression and the lack of a major response to temperature changes suggest that Hsc66 and Hsc20 play an important cellular role(s) under non-stress conditions.

Keywords: ATPase activity; chaperone; co-chaperone; constitutive expression; hsp70; hsp40; purification

The hsp70, or stress-70, proteins comprise a large and widespread family of proteins $\sim 70\text{-kDa}$, which function as ATP-dependent molecular chaperones (reviewed in Gething & Sambrook, 1992; McKay, 1993; Becker & Craig, 1994; Hendrick & Hartl, 1995; Hartl, 1996). Originally discovered as heat shock-induced proteins required for thermotolerance, many members of this family are now known to be constitutively expressed and to function in normal cell activities such as protein folding, processing, and transport. A number of different forms of hsp70 have been identified in eukaryotic organisms, but only a single form, DnaK, has been described in prokaryotes (Georgopoulos, 1977; Bardwell & Craig, 1984; Zylicz & Georgopoulos, 1984). Recently, a gene encoding a 66-kDa protein with sequence similarities to hsp70 proteins was identified in *E. coli* (Kawula & Lelivelt, 1994; Seaton & Vickery, 1994) and subsequently in *Haemophilus influenzae* (Fleischmann et al., 1995). Because expression of the *E. coli* gene is not induced by heat shock, the protein encoded has been designated as a heat shock cognate protein, Hsc66, and the gene as *hscA*. The deduced amino acid sequence of Hsc66 exhibits $\sim 40\%$ identity with DnaK and eukaryotic hsp70 proteins, whereas a somewhat higher sequence identity, generally $>50\%$, is found between hsp70 family

members (Boorstein et al., 1994). The similarities between Hsc66 and hsp70 proteins include signature sequences for the N-terminal ATPase domain as well as the residues in the C-terminal domain believed to function in peptide recognition, suggesting that Hsc66 may function as an ATP hydrolysis-coupled molecular chaperone. Specific sequence differences, however, imply that Hsc66 has different peptide binding properties and that it may be subject to different regulatory mechanisms.

The cellular role of the Hsc66 protein is not known. Hsc66 does not appear to be able to carry out the same functions as DnaK, because deletion of the *dnaK* gene results in a severely growth-restricted phenotype (Bukau & Walker, 1989). The *hscA* gene is located near 54 min on the *E. coli* chromosome (Ta & Vickery, 1992; Ta et al., 1992) and lies in an operon which includes the *fdx* gene, encoding a 12-kDa [2Fe–2S] ferredoxin (Ta & Vickery, 1992), and the *hscB* gene, encoding a 20-kDa protein with a predicted N-terminal region having some sequence similarity to “J-domain” proteins (Kawula & Lelivelt, 1994). The finding that expression of the ferredoxin is translationally coupled to that of Hsc66 suggests the possibility that Hsc66 may have a role in association with the ferredoxin protein (Seaton & Vickery, 1994). The sequence similarities of the predicted 20-kDa gene product to hsp40-related proteins suggests the possibility that Hsc66 may use this protein as a auxiliary, co-chaperone protein; the amino acid sequence of this protein, however, differs significantly from hsp40 proteins both in its small size and its unique C-terminal sequence.

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We report herein the overexpression, purification, and initial characterization of *E. coli* Hsc66 and the *hscB* gene product, designated Hsc20. In addition, antibodies raised against the purified proteins have allowed us to determine their steady state levels and the effects of growth at different temperatures. Our findings are consistent with a role for Hsc66 and Hsc20 as a new molecular chaperone system which functions under non-stress conditions.

Results

Overexpression and purification

The *hscA* and *hscB* genes encoding Hsc66 and Hsc20 were each subcloned into the IPTG-controlled expression plasmid, pTrc99A, to yield pTrcHsc66 and pTrcHsc20. Both constructs were effective in producing high level expression in a number of host strains of *E. coli*. Figure 1 shows SDS-PAGE gels in which IPTG-induced DH5 α cells lacking or harboring the plasmids were analyzed. For cells containing pTrcHsc66 a major band that migrates with an apparent molecular weight ~ 62 k is present and is estimated to comprise $\sim 30\%$ of the total cell protein. For cells containing pTrcHsc20 a major band that migrates with an apparent molecular weight ~ 23 k is present and is estimated to comprise $\sim 50\%$ of the total cell protein.

Cells overproducing Hsc66 were lysed by French press, and the protein was purified by anion-exchange chromatography followed by hydrophobic chromatography. Under the conditions used for anion-exchange chromatography Hsc66 eluted just prior to DnaK, although the two proteins were not completely separated. Complete resolution was obtained during the subsequent reverse-phase chromatography, with Hsc66 eluting at higher salt concentrations than DnaK. The overall yield of purified Hsc66 was ~ 100 mg/L of culture. Attempts to isolate Hsc66 using ATP-affinity chromatography were not successful. In contrast to DnaK (Zylicz et al., 1987) and bovine hsc70 (Welch & Feramisco, 1985) Hsc66 does not bind to agarose columns linked to C-8 of ATP via a 9-carbon spacer; we have not investigated this further, and the structural basis for the failure to bind to the immobilized ATP is not known. Cells overproducing Hsc20 were lysed by French press, and the protein was purified by anion-exchange chromatography followed by size exclusion chromatography. No evidence for tight association of Hsc20 with other proteins was observed, and ~ 0.5 g Hsc20/L of culture was purified with an overall yield $\sim 50\%$.

SDS-PAGE analyses of the final preparations of Hsc66 and Hsc20 are shown in Figure 1. For both proteins a single major band indicative of a high degree of purity was observed. In the case of Hsc66, the apparent molecular weight, approximately 62–63 k, is somewhat lower than expected. Because no indication of proteolytic degradation was observed and because the purified recombinant protein co-migrates with the natural protein in whole cells (see immunoblots below), we assume that full length Hsc66 migrates anomalously fast under the conditions used. N-terminal sequence analysis revealed a single sequence, Ala-Leu-Leu-Gln-Ile-, indicating removal of Met-1 as expected for proteins in which alanine is the penultimate residue (Flinta et al., 1986; Hirel et al., 1989) but no further N-terminal proteolysis. The calculated molecular weight for the mature Hsc66 protein (Seaton & Vickery, 1994; residues 2–616) is 65,516. In the case of Hsc20 the apparent molecular weight, ~ 23 k, is slightly larger than predicted, indicating that Hsc20 migrates slower than expected under these conditions. N-terminal sequence analysis revealed a single sequence,

Fig. 1. SDS-PAGE analysis of Hsc66 and Hsc20 expression and purification. **A:** Lane 1, molecular weight standards; 2, DH5 α cells (40 μ g protein); 3, DH5 α cells harboring pTrcHsc66 plasmid grown for 16 h following IPTG induction (50 μ g protein). Lanes 4–6 contain 1, 3, and 10 μ g purified Hsc66. **B:** Lane 1, DH5 α cells (20 μ g protein); 2, DH5 α cells harboring pTrcHsc20 plasmid grown for 16 h following IPTG induction (20 μ g protein). Lanes 3–5 contain 2, 4, and 10 μ g purified Hsc20. Lane 6, molecular weight standards.

Met-Asp-Tyr-Phe-Thr-, consistent with the *hscB* gene sequence (Kawula & Lelivelt, 1994); retention of Met-1 is typical for *E. coli* proteins having aspartate as the penultimate residue (Flinta et al., 1986; Hirel et al., 1989). The calculated molecular mass for the Hsc20 protein is 20,136 Da.

In order to determine whether Hsc66 and Hsc20 exist in monomeric or oligomeric states, the purified proteins were analyzed by size exclusion chromatography using a Superdex 200 FPLC column. The elution profiles in Figure 2 show a single peak for each protein indicative of a monodisperse sample under the conditions used. The inset to Figure 2 compares the migration positions of

Fig. 2. Gel filtration analysis of Hsc66 and Hsc20. Elution profiles of purified Hsc66 (200 μ g in 50 μ L, 60 μ M) or Hsc20 (100 μ g in 200 μ L, 25 μ M) chromatographed using a Superdex 200 column. Buffer: 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mM DTT, 200 mM NaCl; V_0 indicates the column void volume as determined with blue dextran. The inset shows the elution volumes of Hsc66 and Hsc20 compared to standards (see Materials and methods).

Hsc66 and Hsc20 to proteins of known size. The apparent molecular mass of Hsc66 is $\sim$ 100-kDa, and the apparent Stokes radius corresponds to $\sim$ 44 Å. These values are larger than expected for a symmetrical, monomeric protein of mass 66-kDa but are smaller than expected for a dimer. We interpret these findings to indicate that Hsc66 most likely exists as a monomer in solution and that the protein has an asymmetrical shape that causes it to migrate more rapidly during gel filtration chromatography. Small-angle X-ray scattering studies on bovine hsc70 have indicated that this protein is prolate and elongated (Wilbanks et al., 1995), and Hsc66 may have a similar shape. The apparent molecular mass of Hsc20 is $\sim$ 32-kDa, and the apparent Stokes radius corresponds to $\sim$ 25 Å. These values also are larger than expected for a symmetrical, monomeric protein of mass 20.1-kDa but are smaller than expected for a dimer. We interpret these findings to indicate that Hsc20 also likely exists as a monomer in solution and that the protein has an asymmetrical shape that causes it to migrate more rapidly during size exclusion chromatography. Analysis of the sequence of the C-terminal region of Hsc20 suggests that residues beyond position 90 may fold as a coiled-coil structure (cf. Cohen & Parry, 1990; Lupas, 1996); this region may constitute a distinct domain in addition to the putative N-terminal J-domain and contribute to a non-globular shape.

Spectral properties

The absorption spectra of Hsc66 and Hsc20 (Fig. 3) are consistent with the known amino acid contents of aromatic residues and show no evidence of additional chromophoric groups; the presence of bound nucleotides, for example, would be expected to contribute significant absorption near 260 nm. Using average absorbance values for tryptophan and tyrosine (see Materials and methods) the extinction coefficient for Hsc66 at 280 nm is calculated to be 19.6 (mM $\cdot$ cm) $^{-1}$ and that for Hsc20 at 280 nm is calculated to be 16.8 (mM $\cdot$ cm) $^{-1}$; we have used these values for subsequent determinations of protein concentrations.

Circular dichroism spectra for the 190–250 nm region are presented in Figure 4. The spectrum for Hsc66 resembles that ex-

Fig. 3. Absorption spectra of Hsc66 and Hsc20. Hsc66 concentration: 44 μ M calculated assuming $\epsilon_{280} = 19.6$ (mM $\cdot$ cm) $^{-1}$; Hsc20 concentration: 58 μ M calculated assuming $\epsilon_{280} = 16.8$ (mM $\cdot$ cm) $^{-1}$. Buffer: 0.05 M Tris-HCl, pH 7.4, 1 mM DTT, 0.5 mM EDTA.

pected for a protein having a mixture of α -helical and β -sheet secondary structure. The spectrum is similar to that reported for *E. coli* DnaK (Palleros et al., 1993b) and for bovine hsc70 (Sadis et al., 1990; Park et al., 1993), and comparison to a reference set of proteins of known secondary structure (Yang et al., 1986) suggests an overall content of $\sim$ 28% α -helix and $\sim$ 41% β -sheet. The crystal structure of the N-terminal ATPase domain of bovine hsc70 shows this region of the protein to have 38% α -helix and 30% β -strand (Flaherty et al., 1990), and the crystal structure of the peptide binding domain of DnaK reveals 33% α -helix and 27% β -strand in this region (Zhu et al., 1996); the CD results for Hsc66

Fig. 4. Far UV circular dichroism spectra of Hsc66 and Hsc20. The Hsc66 and Hsc20 concentrations used were 4 and 10 μ M, respectively, and ellipticity data are presented on a mean residue basis. Buffer: 0.05 M Tris-HCl, pH 7.4, 1 mM DTT, 0.5 mM EDTA.

are consistent with an overall secondary structure that is in general similar to these hsp70 proteins. The CD spectrum for Hsc20 resembles that expected for a protein having predominantly α -helical structure. This is consistent with sequence analysis methods (Chou & Fasman, 1974; Garnier et al., 1978) which predict that residues 1–60 are ~50% α -helical and residues 61–171 are ~90% α -helical with little or no β -strand present. This is also consistent with the presence of an α -helical N-terminal J-domain structure similar to that found for *E. coli* DnaJ and human hsp40 J-domain fragments (Hill et al., 1995; Pellecchia et al., 1996; Qian et al., 1996).

Fluorescence spectra are presented in Figure 5. In the case of Hsc66 (Fig. 5A, B) the emission spectra are very sensitive to the excitation wavelength used. The strongest fluorescence emission is observed at 304 nm using 278.5 nm excitation and likely reflects contributions from both tryptophan and tyrosine residues. Excitation at 265 nm yields less intense emission with a λ_{max} at 303 nm suggesting that it originates predominantly from tyrosine residues which do not transfer excitation energy to tryptophan. Excitation at 295 nm selectively excites tryptophan (Hsc66 has a single tryptophan at position 291) and yields a much weaker fluorescence; the emission maximum near 328 nm suggests that Trp-291 is in an environment that is only partially exposed to solvent. In the case of Hsc20 (Fig. 5C), the fluorescence excitation maximum occurs at 283 nm and the emission maximum occurs at 337 nm suggesting that the greatest contributions arise from tryptophan. Two tryptophan residues are present in Hsc20, Trp-58 and Trp-141, but their relative contributions to the observed fluorescence are not known. The emission maximum near 337 nm suggests that emission is from a residue(s) in an environment exposed to solvent.

Expression levels

Purified Hsc66 and Hsc20 were used to elicit antibodies in rabbits, and the antisera were fractionated using Hsc66- and Hsc20-affinity columns. Figure 6A shows Western immunoblots using the affinity-purified antisera to Hsc66. Lanes 1–3 show that Hsc66 is readily detected in cultures of the prototrophic K-12 strain, W3110, the laboratory K-12 derived strain, DH5 α , and the B derived strain, BL21. From comparison of the relative band intensities observed in these lanes with those containing purified Hsc66 in lanes 4–6, we estimate that Hsc66 comprises ~1% of the total cell protein in each of these strains (cells grown overnight to stationary phase at 37°C in LB medium). This is near to the level observed for DnaK (1.4% in glucose rich medium at 37°C; Herendeen et al., 1979) and corresponds to a cellular concentration of ~20 μ M Hsc66. The appearance of a single band corresponding to Hsc66 in the immunoblots of whole cells suggests that anti-Hsc66 does not recognize DnaK, which migrates at a higher apparent molecular weight (Zylicz & Georgopoulos, 1984). This finding, together with our earlier observation that antisera to *E. coli* DnaK do not cross-react with Hsc66 (Seaton & Vickery, 1994), suggests that the major epitopes of the two proteins differ. Our preparation of anti-Hsc66 did not exhibit immunocrossreactivity with purified DnaK or purified bovine BiP (Grp78), even when present in a 20-fold greater amount (data not shown). Thus, the major epitopes of Hsc66 appear to be distinct from those of DnaK and BiP.

Figure 6B shows Western immunoblots using the affinity-purified antisera to Hsc20. Hsc20 is readily detected in cultures of W3110, DH5 α , and BL21 cells grown overnight to stationary phase at 37°C in LB medium. Based on comparisons of the relative band intensities for cells growing at late-log phase at 22–44°C (see below,

Fig. 5. Fluorescence spectra of Hsc66 and Hsc20. A, B: Hsc66 10 μ M in 0.05 M Tris-HCl, pH 7.4, 1 mM DTT, 0.5 mM EDTA. In A, an emission wavelength of 306 nm was used to determine the excitation spectrum; excitation wavelengths used for recording emission spectra are indicated. In B, maximum emission intensities obtained using 265 nm and 295 nm excitation were normalized to the same value to facilitate comparison. C: Hsc20 12 μ M in 0.05 M Tris-HCl, pH 7.4, 1 mM DTT, 0.5 mM EDTA.

Fig. 7C), we estimate that Hsc20 comprises approximately 0.1–0.2% of the total cell protein; this corresponds to a cellular concentration of approximately 6–12 μ M Hsc20 under these conditions.

Fig. 6. Immunoblot analyses of Hsc66 and Hsc20 expression. **A:** detection of Hsc66 in W3110, DH5 α , and BL21 cultures grown at 37°C in LB to stationary phase. Lanes 1–3: W3110 cells, DH5 α cells, and BL21 cells (each 8 μ g protein). Lanes 4–6: 60, 80, 100 ng purified Hsc66. **B:** detection of Hsc20 in W3110, DH5 α , and BL21 cultures grown at 37°C in LB to stationary phase. Lanes 1–3, W3110 cells, DH5 α cells, and BL21 cells (each 24 μ g protein).

Antisera to Hsc66 and Hsc20 were also used to investigate possible changes in the steady state levels of the proteins in response to temperature shifts. Levels of mRNA encoding Hsc66 do not change following heat shock (Kawula & Lelivelt, 1994; Seaton & Vickery, 1994; Lelivelt & Kawula, 1995) but have been reported to increase following a “cold-shock” shift from 37°C to 10°C (Lelivelt & Kawula, 1995). We shifted W3110 cells growing in LB at 37°C to 10°C, 22°C, 37°C, or 44°C at an initial cell density corresponding to $A_{600} = 0.5$ and allowed cell growth to continue to late-log phase ($A_{600} \sim 1.5$) for each culture. As shown in Figure 7A, similar amounts of immunoreactive Hsc66 appeared to be present in cells grown at all temperatures. Because of the extended time period required for cell growth at 10°C, we also cultured cells for a shorter period, 5 hr, following the temperature shift and compared levels of Hsc66 present at 37°C and 10°C. Immunoblots from these cultures are shown in Figure 7B. Again, no major difference in the amount of Hsc66 in cold-stressed cells compared to that at 37°C is apparent.

Figure 7C compares the level of Hsc20 present in W3110 cells grown to mid-late log phase in rich medium at several temperatures. No increase in Hsc20 level is observed following heat shock at 44°C, but a small increase (~ 2 -fold) in the amount of Hsc20 is observed in cultures grown at 10°C compared to cultures grown at 22°C, 37°C, or 44°C. Lelivelt and Kawula (1995) observed increased *hscB* mRNA levels, as measured by primer extension, in cells shifted from 37°C to 10°C for three hours, and our finding of an increase in the level of Hsc20 is consistent with enhanced expression at low temperatures.

ATPase activity

The ATPase activity of purified Hsc66 was initially determined by a colorimetric assay for phosphate release (Lanzetta et al.,

Fig. 7. Effect of temperature on expression of Hsc66 and Hsc20. **A:** cultures of W3110 cells growing in LB at 37°C ($A_{600} = 0.5$) were shifted to various temperatures and grown to similar cell densities ($A_{600} \sim 1.5$). Lane 1, 10°C (24 h growth); 2, 22°C (6 h growth); 3, 37°C (3 h growth); 4, 44°C (1.5 h growth). Cells equivalent to 8 μ g protein were loaded in each lane. **B:** cultures of W3110 cells were shifted from 37°C ($A_{600} = 0.2$) and grown an additional 5 h at 37°C or 10°C. Cells equivalent to 6 μ g protein were loaded in each lane. **C:** Lanes 1 and 2, 30 and 40 ng purified Hsc20. Lanes 3–6, W3110 cells from cultures grown to $A_{600} = 1.5$ at 10°C, 22°C, 37°C, and 44°C (each 24 μ g protein).

1979) which allowed measurements over a broad range of temperatures. Because of the low catalytic rates, experiments were conducted using high concentrations of Hsc66 (5–40 μ M) and ATP (400 μ M). These Hsc66 concentrations are near to the cellular level (~ 20 μ M; see above). The ATP concentration used is below that in rapidly growing cells (~ 3 mM; Neuhard & Nygaard, 1987), but assuming that Hsc66, like DnaK, has a low K_m for ATP, the rates observed should reflect maximal turnover numbers for the solution conditions employed. Rates observed for DnaK using these conditions are in good agreement with those obtained by others using lower enzyme and substrate concentrations (McCarty & Walker, 1991; Palleros et al., 1993a). Figure 8 shows the variation in rates of ATP hydrolysis observed for Hsc66 and DnaK over the range 10–55°C. Hsc66 exhibits greater activity than DnaK (0.6 min^{-1} vs. 0.1 min^{-1} at pH 7.5, 37°C), and the Hsc66 ATPase rates are also somewhat higher than those typical of other hsp70 proteins (McCarty & Walker, 1991; Palleros et al., 1993a; Kamath-Loeb et al., 1995; O'Brien & McKay, 1995; Wei et al., 1995; Ziegelhoffer et al., 1995). The increased activity of Hsc66 at high temperatures (approximately two-fold greater at 44°C than at 37°C) suggests that it is active in the cell under conditions of heat stress; however, activity declined at temperatures greater than 50°C suggesting that Hsc66 is less thermostable than DnaK. At low temperatures the ATPase activity Hsc66 activity is reduced (eight-fold lower at 10°C compared to 37°C), suggesting that it is likely to function less well under conditions of cold stress.

The effect of Hsc20 on the ATPase activity of Hsc66 was investigated using a coupled enzyme assay for phosphate release

Fig. 8. Temperature dependence of the intrinsic ATPase activity of Hsc66. The ATP concentration was 400 μ M, and the buffer contained 0.1 M Hepes, pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT. The concentrations of Hsc66 and DnaK varied from 5–40 μ M (see Methods).

(Webb, 1992) which provided greater sensitivity and allowed use of lower concentrations of Hsc66; for these studies experiments were carried out at pH 7.0 and at 25°C. Figure 9 shows the effect of a 2:1 ratio of Hsc20 (4 μ M) on the rate of ATP hydrolysis catalyzed by Hsc66 (2 μ M). Under these conditions Hsc20 increases the specific ATPase activity of Hsc66 from 0.135 to 0.312 mol ATP hydrolyzed/mol Hsc66/min corresponding to a 2.3-fold

Fig. 9. Effect of Hsc20 on the ATPase activity of Hsc66. Time course of ATP hydrolysis catalyzed by Hsc66 (2 μ M) at 25°C in the absence (open symbols) or presence (closed symbols) of Hsc20 (4 μ M). The ATP concentration was 400 μ M, and the buffer contained 0.1 M Hepes, pH 7.0, 150 mM KCl, 1 mM $MgCl_2$, 1 mM DTT.

increase (Hsc20 alone does not exhibit ATPase activity, data not shown). Experiments were carried out over a range of concentrations of Hsc20 to determine the affinity of Hsc66 for Hsc20. The results are shown in Figure 10 and are plotted as the increase in ATPase activity relative to the rate in the absence of Hsc20 as a function of Hsc20 concentration. Assuming 1:1 stoichiometry for the binding of Hsc20 to Hsc66, hyperbolic saturation curves are obtained when the data are corrected for the amount of Hsc20 bound. Extrapolation to saturating levels of Hsc20 indicates a maximal stimulation of 3.8-fold, with half-maximal stimulation at 4.5 μ M Hsc20.

Discussion

The expression systems described herein afford good yields of Hsc66 and Hsc20; both proteins appear to be stable and accumulate to high levels without apparent toxicity. The level of Hsc20 produced, approximately half of the total cell protein, is particularly impressive and corresponds to ~1 g Hsc20/L cells after overnight growth in rich medium. The final yields of purified Hsc66 and Hsc20, ~0.1 g and 0.5 g/L, respectively, provide sufficient amounts of the proteins for biophysical studies. The general properties of Hsc66 were found to be similar to those of hsp70 proteins: Hsc66 exists predominantly as a monomer in solution,

Fig. 10. Effect of Hsc20 concentration on the ATPase activity of Hsc66. Data are reported as the increase in basal ATPase activity rates (0.135 mol P_i released/mol Hsc66/min measured in the absence of Hsc20) at 25°C. The concentration of Hsc66 used was 2 μ M and concentrations of Hsc20 were varied from 0.8–40 μ M. The ATP concentration was 400 μ M, and the buffer contained 0.1 M Hepes, pH 7.0, 150 mM KCl, 1 mM $MgCl_2$, 1 mM DTT. The concentration of free Hsc20 was calculated assuming 1:1 binding stoichiometry with Hsc66 using the equation

$$Hsc20_{free} = Hsc20_{total} - \frac{\Delta V \cdot E}{\Delta V_{max}}$$

where $Hsc20_{total}$ is the concentration of added Hsc20, ΔV is the observed rate change at $Hsc20_{total}$, E is the total concentration of Hsc66, and ΔV_{max} is the rate change extrapolated to infinite Hsc20 concentration. The curve shown represents a calculated hyperbolic saturation function assuming a maximal stimulation of 3.8-fold and half-maximal stimulation at 4.5 μ M Hsc20.

size exclusion chromatography suggests an asymmetric shape, and far-UV circular dichroism spectra are consistent with mixed α/β secondary structure. Fluorescence spectra of Hsc66 are distinct due to differences in specific positions of aromatic residues, and the unique fluorescence arising from Trp-291 may provide a useful intrinsic probe for monitoring conformation in this region of the protein. The general properties of Hsc20 were also determined and revealed that it is monomeric in solution, has an asymmetric shape which may reflect a multidomain structure, and appears to have predominantly α -helical secondary structure. Fluorescence from Trp-58 and/or Trp-141 of Hsc20 suggests a surface location exposed to the solvent.

A hallmark of hsp70-type chaperones is a low intrinsic ATPase activity. Purified Hsc66 exhibits ATPase activity with a turnover number 0.6 min^{-1} (37°C , pH 7.5), a value somewhat higher than typically observed for hsp70s and ~ 6 -fold greater than the intrinsic activity of *E. coli* DnaK assayed under similar conditions. The temperature-activity profile for Hsc66 is similar to that of DnaK with a large increase in ATPase activity observed over the range 10 – 50°C . These findings suggest that Hsc66 is likely to function as an ATP-coupled chaperone in a manner similar to hsp70s. The low sequence identity of Hsc66 compared to other hsp70s, however, suggests that Hsc66 may exhibit different functional properties. Sequence differences in the N-terminal ATPase domain could make Hsc66 subject to different types of regulation, and differences in the C-terminal peptide binding domain seem likely to result in different peptide binding properties for Hsc66 compared to DnaK. The fact that disruption of the DnaK gene in *E. coli* results in a severely growth-restricted phenotype despite the high constitutive levels of Hsc66 (see below), also indicates that the peptide substrate specificity of Hsc66 is different from that of DnaK. Peptide substrates for Hsc66 have not been identified, and the exact physiological role of Hsc66 is not known. Disruption of the *hscA* gene, unlike the *dnaK* gene, does not have dramatic effects on viability (Lelivelt & Kawula, 1995) or on growth in rich media (D.T. Ta and L.E. Vickery, unpublished). Detailed characterization of the phenotype of *hscA*[−] strains may provide useful insights into its function(s).

Hsc20 stimulates the intrinsic ATPase activity of Hsc66. The maximal increase observed, ~ 4 -fold at 25°C , is less than the maximal stimulation of DnaK intrinsic ATPase activity by DnaJ which is ~ 10 -fold (Jordan & McMacken, 1995; McCarty et al., 1995). Because of the higher intrinsic activity of Hsc66 compared to DnaK, however, the maximal Hsc20-stimulated ATPase activity of Hsc66 ($\sim 0.5 \text{ min}^{-1}$ at 25°C) is in the same range as the maximal DnaJ-stimulated ATPase activity of DnaK. These findings suggest that Hsc20 has functions similar to DnaJ and other hsp40-type co-chaperones. The size and amino acid sequence of Hsc20, however, differ significantly from the hsp40 proteins. Sequence similarities are limited to the putative J-domain at the N-terminus. The highly conserved J-motif signature sequence His-Pro-Asp is found at positions 32–34 of Hsc20, but the overall sequence identity in this region is only $\sim 15\%$ (N-terminal 70 residues) compared to $\sim 50\%$ identity observed among hsp40s. The C-terminal region of Hsc20 is significantly smaller (~ 12 -kDa) than found in hsp40 proteins (~ 32 -kDa) and lacks the glycine-rich segment and cysteine-rich/zinc-finger domain (reviewed by Caplan et al., 1993; Silver & Way, 1993; Cyr et al., 1994; Rassow et al., 1995). Thus Hsc20 appears to represent a new type of J-protein and a new class of co-chaperone, and it seems likely that it will participate in its own regulatory interactions distinct from those of hsp40-type co-chaperones.

Using affinity-purified antisera to Hsc66 and Hsc20 we also measured their cellular levels and their responses to temperature changes. Both proteins were found to be constitutively expressed and abundant in *E. coli* grown in rich media. The level of Hsc66 corresponds to $\sim 1\%$ of the total cell protein and to a cellular concentration $\sim 20 \mu\text{M}$. These values are comparable to the basal level of DnaK in cells grown at 37° (Herendeen et al., 1979), and suggest that Hsc66 has important cellular functions during growth at normal temperatures. No major changes were observed in the levels of Hsc66 as a result of temperature shifts either to 44°C or to 10°C . The lack of induction by heat shock is in accord with previous studies (Kawula & Lelivelt, 1994; Seaton & Vickery, 1994; Lelivelt & Kawula, 1995). The lack of a detectable change in the level of Hsc66 following cold shock, however, appears counter to the finding that transcription of the *hscA* gene is elevated for several hours following cold shock (Lelivelt & Kawula, 1995). A possible explanation is that the increase in transcription and any increase in translation that occur at low temperature do not significantly alter the steady state level of Hsc66, which is already quite high.

The level of Hsc20, like that of Hsc66, did not change appreciably following heat shock, but a small increase (estimated ~ 2 -fold) was observed in cultures shifted to 10°C . Lelivelt and Kawula (1995) observed increased *hscB* mRNA levels, as measured by primer extension, in cells shifted from 37° to 10°C , and our finding of an increase in the level of Hsc20 are consistent with enhanced expression of Hsc20 at low temperatures. Based on comparisons of the relative band intensities for cells grown at 22 – 44°C with those containing purified Hsc20, we estimate that constitutive levels of Hsc20 are approximately 0.1 – 0.2% of the total cell protein corresponding to a cellular concentration of approximately 6 – $12 \mu\text{M}$ Hsc20 under these conditions. This concentration is approximately half that estimated for Hsc66 for a ratio of Hsc20:Hsc66 $\sim 0.5:1$. From studies on the effect of varying concentrations of Hsc20 on the ATPase activity of Hsc66, half-maximal stimulation of Hsc66 ATPase was observed when the concentration of free Hsc20 was $\sim 5 \mu\text{M}$ assuming $1:1$ binding stoichiometry. Cellular levels of Hsc20 thus appear to be sufficiently high for significant binding to Hsc66. Because levels of Hsc66 are greater than Hsc20, however, Hsc66 will not be saturated with Hsc20; for this reason changes in the level of Hsc20, such as observed under cold stress, could have effects on Hsc66 activity. Each molecule of Hsc20 may interact with multiple Hsc66 molecules during catalysis depending on the relative rates of ATP hydrolysis and Hsc20 association and dissociation; as with other hsp70-type systems these rates may also be affected by the presence of peptide substrates which interact with Hsc66.

Hsc66 and Hsc20 represent new members of the hsp70-type chaperone/co-chaperone family and comprise a second prokaryotic hsp70 system in addition to DnaK/DnaJ. The exact cellular role(s) of Hsc66 and Hsc20 remain to be determined, but the relatively high constitutive level of expression and the lack of major changes in response to thermal stress suggest general "house-keeping" metabolic function(s) in addition to any role(s) they may play in thermotolerance. The occurrence of Hsc66 and Hsc20 in organisms other than *E. coli* has not been described, but the recent sequencing of the complete genome of *H. influenzae* (Fleischmann et al., 1995) has revealed that this organism also possesses genes corresponding to *hscA* and *hscB* and that they are organized in a similar colinear manner suggesting that this molecular chaperone system may be widely distributed.

Materials and methods

Materials

E. coli W3110 was from the American Type Culture Collection (ATCC number 27325), DH5 α F'IQ cells were from GibcoBRL, and BL21(DE3)pLysS cells were from Novagen. The DnaK expression plasmid, pJM2 (McCarty & Walker, 1991), was generously provided by G.C. Walker, and purified BiP(GRP78) was a gift from Stressgen Biotechnologies Corp. Enzymes for DNA manipulation were obtained from Boehringer-Mannheim Corp., New England Biolabs, Inc., or United States Biochemical Corp. Synthetic oligonucleotides were obtained from Operon Technologies. Bacterial growth media components were from Difco, and other reagents were from Sigma Chemical Co.

Expression and purification methods

The vector used to overexpress Hsc66, pTrcHsc66, was constructed from pDT10 (Seaton & Vickery, 1994) and pTrc99A (Pharmacia). The *EcoRI*/*NsiI* fragment of pDT10 containing *hscAfdx* was ligated into pTrc99A, which had been digested with *EcoRI* and *PstI* to give pTrcDT10. A 790 bp 5' fragment of the *hscA* gene was amplified by PCR using *Taq* DNA polymerase (United States Biochemical) to introduce a *NcoI* site at Met-1 and include the *NruI* site at 739bp. The PCR product was digested with *NcoI* and *NruI* and ligated into *NcoI*/*NruI*-digested pTrcDT10. This produced pTrcHsc66 allowing for expression of Hsc66 (and ferredoxin) under IPTG (isopropylthio- β -D-galactoside) control. The vector used for overexpressing Hsc20, pTrcHsc20, was constructed by cloning the *hscB* gene into pTrc99A (Pharmacia). The coding region of *hscB* was amplified from genomic DNA isolated from *E. coli* K-12 strain W3110 by PCR using *Pfu* DNA polymerase (Stratagene) and primers designed to introduce *NcoI* restriction sites at positions corresponding to Met-1 of Hsc20 and of Hsc66. The PCR product was digested with *NcoI* and ligated into pTrc99A which had been digested with *NcoI* and treated with calf intestinal phosphatase to minimize reclosure. DH5 α cells were transformed with the resulting pTrcHsc20 plasmid, and colonies containing the *hscB* gene in the correct orientation were identified by PCR analysis. The *hscB* gene in pTrcHsc20 was sequenced and was found to be identical to that reported by Kawula and Lelivelt (1994).

DH5 α cells transformed with pTrcHsc66 or pTrcHsc20 were grown in Terrific Broth at 37°C, induced with 0.5 mM IPTG at $A_{600} \approx 1$, and grown ~16 h to allow expression. Cells were harvested by centrifugation, frozen, thawed, and lysed by French press in TED buffer (50 mM Tris-HCl, pH 7.4, 1 mM DTT (dithiothreitol), 0.5 mM EDTA,) containing 0.4 mM phenylmethylsulfonyl fluoride (PMSF) to inhibit proteolysis. The soluble supernatant fluid following centrifugation at $37k \times g$ was used for purification, and all subsequent chromatography was carried out in TED buffer at 4°C. Crude cell lysates containing either Hsc66 or Hsc20 were initially purified using DEAE-cellulose (DE52, Whatman). Soluble extracts were applied to the column and washed with several column volumes of TED containing 0.4 mM PMSF. Hsc66 was eluted using a linear gradient of 50 to 150 mM NaCl, and fractions were analyzed by gel electrophoresis, combined, dialyzed against TED, and applied to a DEAE-Sepharose column (Pharmacia). After washing, Hsc66 was eluted using a linear gradient of 50 to 150 mM NaCl. Gel analysis at this stage showed that Hsc66 eluted slightly earlier than DnaK, but was not completely separated. Fractions enriched in Hsc66 were combined, adjusted to

20% saturation with ammonium sulfate, and applied to a Phenyl Sepharose column (Pharmacia). After washing with 20% ammonium sulfate, Hsc66 was eluted with a linear gradient decreasing from 20–10% ammonium sulfate; DnaK remained bound to the column under these conditions. The final preparation of Hsc66 was concentrated by ultrafiltration to >30 mg protein/mL, frozen in liquid nitrogen, and stored at –70°C. Hsc20 was eluted from the initial DEAE-cellulose column using a linear gradient from 0 to 50 mM NaCl followed by isocratic elution with 50 mM NaCl. Fractions were analyzed by gel electrophoresis, pooled, concentrated by ultrafiltration, and subjected to size exclusion chromatography using Sephadex G100 (Pharmacia). Fractions appearing homogeneous were combined and concentrated by ultrafiltration to >30 mg protein/mL, frozen in liquid nitrogen, and stored at –70°C.

DnaK was purified from JM109 cells harboring the plasmid pJM2 (McCarty & Walker, 1991). Expression was induced with IPTG as for Hsc66, and a similar purification procedure was used. DnaK completely separated from Hsc66 during the hydrophobic chromatography step on Phenyl Sepharose and was eluted using a gradient of 20–5% ammonium sulfate. The final preparation gave a single band on SDS-PAGE and was stored as for Hsc66.

ATP-affinity chromatography of Hsc66 and DnaK was carried out as described by others (Welch & Feramisco, 1985; Zylitz et al., 1987) using ATP coupled at C-8 to agarose through a 9-atom spacer (Sigma Chem. Co., A2767). The column was equilibrated with 25 mM Hepes, pH 8.0, 3 mM magnesium chloride, 10 mM sodium chloride, 10 mM 2-mercaptoethanol, and 1 mM EDTA. After sample application the column was washed with 0.3 M sodium chloride, and protein eluted with 10 mM ATP. Under these conditions purified DnaK bound to the column and could be eluted with ATP, whereas purified Hsc66 failed to bind to the column.

Antisera

Two rabbits were each injected five times with 100 μ g purified Hsc66 or 100 μ g Hsc20 by Bethyl Laboratories, and serum from several bleedings of both rabbits for each protein were combined. Hsc66 and Hsc20 affinity columns were prepared by reacting 30 mg purified Hsc66 or Hsc20 with 8 ml cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Antisera were diluted 5-fold with 50 mM Tris-HCl, pH 7.4, and applied to the columns. The columns were washed with the same buffer containing 0.5 M sodium chloride and then with 0.1 M glycine-HCl, pH 3.5. Affinity purified antisera were eluted with 0.1 M glycine-HCl, pH 3.0 and rapidly neutralized with Tris base.

Analytical methods

SDS-PAGE was carried out according to Laemmli (1970), and Western immunoblotting was carried out as described by Towbin et al. (1979) using enhanced chemiluminescence detection (Amersham). N-terminal amino acid sequencing employed Edman degradation (A. Henschen-Edman, Univ. Calif., Irvine).

Estimations of the molecular size of Hsc66 and Hsc20 were made using a Superdex 200 HR 10/30 column on an FPLC system (Pharmacia). The standards (kDa) used included thyroglobulin (669), ferritin (440), catalase (232), aldolase (158), albumin (69.2), ascorbate peroxidase (57.5), ovalbumin (43), maltose binding protein (40.4), rhodanese (33.3), chymotrypsinogen (25), ribonuclease A (13.7), and cytochrome c (12.4).

Absorption spectra were recorded using a Cary 1 spectrometer. The extinction coefficient of Hsc66 at 280 nm, $\epsilon_{280} = 19,600 \text{ (M}\cdot\text{cm)}^{-1}$, was calculated based on 1 tryptophan and 10 tyrosine residues (Seaton & Vickery, 1994) using average absorptivities for tryptophan and tyrosine of 5,600 and 1,400 $\text{(M}\cdot\text{cm)}^{-1}$, respectively (Gill & von Hippel, 1989; Mach et al., 1992; Pace et al., 1995). The extinction coefficient of Hsc20 at 280 nm, $\epsilon_{280} = 16,800 \text{ (M}\cdot\text{cm)}^{-1}$, was similarly calculated based on an amino acid composition having 2 tryptophan and 4 tyrosine residues (Kawula & Lelivelt, 1994). Circular dichroism spectra were recorded with a Jasco 720 spectropolarimeter using a spectral band pass of 2 nm and a cell pathlength of 0.5 mm. Fluorescence spectra were recorded using a SLM-Aminco 8100 fluorometer with an excitation spectral band width of 2 nm and an emission spectral band width of 4 nm in a 1 cm cell.

ATPase assays

ATPase activities were determined by measuring phosphate release using either a colorimetric assay as described by Lanzetta et al. (1979) or a coupled enzyme assay (Webb, 1992) with the EnzChek phosphate assay kit as recommended by the manufacturer (Molecular Probes). The concentration of ATP (disodium salt, Sigma Chemical Co.) in all assays was 400 μM . For temperature dependence studies the colorimetric assay was used, and reaction mixtures contained 0.1 M Hepes, pH 7.5 (adjusted with sodium hydroxide), 150 mM KCl, 5 mM MgCl_2 , 1 mM DTT, in 1 ml. Samples were pre-equilibrated for 5 min at the reaction temperature prior to addition of ATP; the concentrations of Hsc66 were 40 μM (10°, 20°, 22°, 55°C), 20 μM (30°C), 10 μM (37°, 40°, 44°C), or 5 μM (50°C); DnaK concentrations were 40 μM (10–37°C), 20 μM (40°C), or 5 μM (50°, 55°C). Reactions were stopped at various times by addition of 5 μL of 50% trichloroacetic acid to a 50 μL aliquot; samples were centrifuged $14,000 \times g$ for 3 min to remove protein prior to phosphate determination. For studies on the effect of Hsc20 on Hsc66 ATPase activity using the EnzChek kit, reactions mixtures contained 0.1 M Hepes, pH 7.0, 150 mM KCl, 1 mM MgCl_2 , and 1 mM dithiothreitol, in 1 mL. For all assay conditions reported reaction rates were directly proportional to enzyme concentration.

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