Self-Association of the Molecular Chaperone HSC70†

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Received June 13, 1995; Revised Manuscript Received September 15, 1995[⊗]

ABSTRACT: The self-association properties of the molecular chaperone HSC70 have been analyzed by a wide range of biochemical and biophysical techniques. Nondenaturing gel electrophoresis and cross-linking studies show the presence of multiple species going from monomer to at least trimer. Size-exclusion chromatography gives two overlapping peaks, a major one corresponding to species having the molecular mass of monomer (70 kDa) and a minor broad one corresponding to species with a molecular mass range of 150−300 kDa. Progressive dilution of the protein leads to an increase in the size of the monomer peak at the expense of that of the oligomeric peak, thus indicating a concentration-dependent chemical equilibrium. Sedimentation velocity reveals the presence of three species, whose proportions were dependent on concentration, but whose sedimentation coefficients, $s_{20,w}$, of 4.3, 6.6, and 8.5 S did not vary with concentration, indicative of a slowly equilibrating system. Sedimentation equilibrium studies confirmed these results and showed a dissociation into monomers at low concentrations and an association into dimers and trimers at high concentrations. The multiple sedimentation equilibrium datasets, obtained at various initial loading concentrations as well as different rotor speeds, were fitted to a single set of equilibrium constants by a monomer−dimer−trimer association model in which the association constants for the monomer−dimer and dimer−trimer equilibrium were respectively $K_{1-2} = 1.1 \times 10^5\ M^{-1}$ and $K_{2-3} = 0.9 \times 10^5\ M^{-1}$. Interestingly, an isodesmic, indefinite type of association describes the data almost equally well with a single constant of $1.2 \times 10^5\ M^{-1}$. Altogether, these results indicate that HSC70 slowly and reversibly self-associates in solution, likely in an unlimited fashion, and might have implications for the chaperone function of this protein.

The 70 kDa heat shock cognate protein (HSC70),[1] a constitutively expressed member of the highly conserved 70 kDa heat shock protein family (HSP70), plays an essential role in several cellular processes such as protein folding, assembly, and transport, and has been shown to be required for cell viability (Craig et al., 1987; Werner-Washburne et al., 1987). HSC70 is thought to act as a molecular chaperone, by transiently binding to presumably hydrophobic regions of polypeptide chains, thereby preventing incorrect intra- and intermolecular interactions, and dissociating from the bound polypeptide upon ATP binding [see reviews by Hendrick and Hartl (1993), McKay (1993), McKay et al. (1994), and Hightower et al. (1994)].

HSC70 consists of two domains, an amino-terminal ATPase domain which binds and hydrolyzes ATP and a carboxy-terminal domain involved in the binding of the protein substrates (Chappell et al., 1987; Wang et al., 1993). Although the three-dimensional structure of the entire protein is unknown, the isolated amino-terminal fragment of 44 kDa has been crystallized and its structure solved to a resolution of 2.2 Å (Flaherty et al., 1990). However, only the secondary structure topology of the carboxy-terminal domain (residues 385−543), as determined by NMR methods, is available (Morshauser et al., 1995).

The protein, purified originally as an uncoating ATPase (Schlossman et al., 1984; Ungewickell, 1985; Chappell et al., 1986), binds tightly to ATP and ADP (Schmid et al., 1985; Palleros et al., 1991; Gao et al., 1993; Ha & McKay, 1994). In fact, these nucleotides bind so strongly that the protein has an ADP bound to its active site, and is consequently in the ADP form, after the usual preparation method involving an ATP elution step from ATP−agarose affinity columns (Schmid et al., 1985; Gao et al., 1993, 1994). However, HSC70 has a weak intrinsic ATPase activity (Sadis & Hightower, 1992; Wilbanks et al., 1994), that is stimulated 2−5-fold by clathrin light chains (Braell et al., 1984; DeLuca-Flaherty et al., 1990), synthetic peptides (Flynn et al., 1989; Lam & Calderwood, 1992; Wang & Lee, 1993; Fourie et al., 1994; Kwon & Churchich, 1994), unfolded proteins (Palleros et al., 1991; Sadis & Hightower, 1992; Benaroudj et al., 1994), and other heat shock proteins such as DnaJ homologs (Cyr et al., 1992; Cheetham et al., 1994).

Previous studies using nondenaturing gel electrophoresis (Kim et al., 1992), size-exclusion chromatography, and chemical cross-linking (Schlossman et al., 1984; Schmid et al., 1985; Guidon & Hightower, 1986; Palleros et al., 1991; Benaroudj et al., 1994) have shown that HSC70 self-associates in solution to form dimers and trimers. Consistent with these observations, oligomers of various sizes have been observed by electron microscopy (Heuser & Steer, 1989). Most importantly, members of the HSP70 family studied to date such as the bacterial DnaK, plant HSC70, the bovine endoplasmic reticulum resident BiP, or the human heat shock inducible HSP70 self-associate to form multiple species (Freiden et al., 1992; Carlino et al., 1992; Palleros et al.,

† This work was supported in part by the Association pour la recherche sur le cancer (ARC) and the Ligue Nationale contre le cancer (FNCLCC). N.B. is supported by an ARC predoctoral fellowship.

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⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1995.

[1] Abbreviations: HSC70, 70 kDa heat shock cognate protein; HSP70, 70 kDa heat shock protein.

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1993; Blond-Elguindi et al., 1993; Brown et al, 1993; Anderson et al., 1994; Schoenfeld et al., 1995; Bhattacharaya et al., 1995), suggesting that self-association is a general, conserved structural feature of the HSP70 family that must be important for function. In spite of these observations, the oligomerization properties of the protein have never been rigorously addressed, and the exact nature as well as the quantitative distribution of the various species present in solution never examined.

In this work, gel electrophoresis, cross-linking, size-exclusion chromatography, and analytical ultracentrifugation have been used to assess the thermodynamic properties of HSC70 self-association and to define the mechanisms and equilibrium constants involved in this process. Possible implications of self-association for the chaperone function of HSC70 are discussed.

MATERIALS AND METHODS

Materials. ATP and ATP−agarose (C8 linked) were purchased from Sigma while other liquid chromatography materials and FPLC products were from Pharmacia. Electrophoresis supply was from BioRad and all other chemicals from Merck.

Protein Expression and Purification. Recombinant HSC70 was expressed and purified as described previously (Benaroudj et al., 1994), except that following the ATP−agarose affinity column step, fractions containing HSC70 were pooled, concentrated by ultrafiltration using a YM10 membrane on an ultrafiltration cell (Amicon), and applied onto a PD10 desalting column, equilibrated with 20 mM Tris-HCl, pH 7.5, 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, and 1 mM β-mercaptoethanol, to remove free nucleotide. However, after this step, the protein still retains an ADP tightly bound at the active site, due to the strong binding of this nucleotide (K_d = 10 nM), and is therefore in the ADP-bound form (Schmid et al., 1985; Gao et al., 1993, 1994). The protein was then snap-frozen and stored at −80 °C.

Protein concentration was determined by the method of Lowry using bovine serum albumin as a standard or by using an extinction coefficient at 280 nm of 0.62 cm^2 mg^{-1} (Greene & Eisenberg, 1990) for a 1 mg/mL protein solution.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) in denaturing conditions (SDS) was carried out in 0.75 mm thick 12% acrylamide slab gels according to Laemmli (1970). Gels were run using the Mini-Protean II apparatus from BioRad. PAGE in native conditions was performed on 6% acrylamide slab gels according to Kim et al. (1992).

For cross-linking studies, SDS−PAGE was carried out in 3−7% acrylamide slab gels according to Laemmli (1970). Cross-linked phosphorylase *b* (Sigma) and molecular weight markers (BioRad) were used as molecular weight standards. Staining of all gels was with Coomassie brillant blue R250.

Cross-Linking Studies. Protein samples diluted in 10 mM potassium phosphate buffer, pH 7.0, to the concentrations indicated in the text were incubated at room temperature, for 1 min, with glutaraldehyde at a final concentration of 0.1%, and the reaction was stopped by the addition of 60 mM $NaBH_4$. After 10 min incubation at room temperature, proteins were precipitated with trichloroacetic acid and analyzed by SDS−PAGE as described above.

Size-Exclusion Chromatography. Size-exclusion chromatography was carried out at room temperature, using an FPLC system (Pharmacia), on a Superose 12 (preparative grade) 10/30 column equilibrated with 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM β-mercaptoethanol. Elution was performed using the same buffer, fractions of 0.5 mL were collected at a flow rate of 0.2 mL/min, and absorbance was measured at 280 nm. The column was calibrated with a high and low molecular weight calibration kit from Pharmacia. For the ATP experiments, 100 μM ATP was present in all buffers.

Sedimentation Velocity. Sedimentation velocity experiments were performed at 20 °C on a Beckman Optima XL-A analytical ultracentrifuge equipped with a An Ti 60 titanium four-hole rotor with two-channel 12 mm path-length centerpieces. Sample volumes of 400 μL were centrifuged at 60 000 rpm, and radial scans of absorbance were taken at 10 min intervals. Data analysis was performed using the computer programs XLAVEL and XLA-VELOC supplied by Beckman and SVEDBERG (Philo, 1994) provided by John Philo. Hydrodynamic parameters of HSC70 molecular species were evaluated according to Waxman et al. (1994), using the program AXIAL provided by Les Holladay. The molecular mass of the monomer, 70 870 Da, and the partial specific volume, $\bar{v}$, at 20 °C, 0.735 cm^3/g, were calculated from the amino acid composition. The degree of hydration of the protein, 0.46 g of H_2O/g of protein, was estimated, based on amino acid composition, by the method of Kuntz (1971) according to Laue et al. (1993). However, since this value is calculated for a totally unfolded protein in which all the amino acids are exposed to solvent, and tends therefore to be overestimated, a correction factor is necessary to account for internal residues that are not accessible to solvent. A correction factor of 0.7 is obtained by comparing degrees of hydration for several proteins in their folded state to that based on their amino acid composition (Lin et al., 1991). Thus, a degree of hydration of 0.32 g of H_2O/g of protein was used for all calculations.

Sedimentation Equilibrium. Sedimentation equilibrium experiments were carried out at 4 °C using three loading concentrations (0.3, 0.6, and 1.2 mg/mL) and three rotor speeds (8000, 12 000, and 16 000 rpm). Radial scans of the absorbance at 280 nm were taken at 2 h intervals, and samples were judged to be at equilibrium by the absence of systematic deviations in overlayed successive scans and when a constant average molecular mass (M_w) was obtained in plots of M_w versus centrifugation time. The partial specific volume of HSC70, $\bar{v}$, was calculated to be 0.729 mL/g at 4 °C according to Laue et al. (1993) and the solvent density taken as 1.00 g/mL. Multiple datasets were analyzed using the appropriate functions by nonlinear least-squares procedures provided in the Beckman Optima XL-A software package (McRorie & Voelker, 1993).

For data analysis according to discrete self-association models, the general equation was used:

$$C(r) = \delta + C_{10} \exp[\sigma(r^2 - r_0^2)] + \sum_{N>1} C_{10}^N K_N \exp[N\sigma(r^2 - r_0^2)]$$

where $C(r)$ is the total concentration at radius r, δ is the base line offset, C_{10} is the monomer concentration at the reference radius r_0, N is the stoichiometry of the reaction,

and K_N is the equilibrium association constant. σ is the reduced molecular weight, and is defined as

$$\sigma = M_1(1 - \bar{v}\rho)\omega^2/2RT$$

where M_1 is the monomer molecular weight, $\bar{v}$ is the partial specific volume, ρ is the solvent density, ω is the angular velocity of the rotor, R is the gas constant, and T is the absolute temperature of the sedimentation equilibrium experiment. The monomer molecular mass of 70 870 Da used in these studies is calculated from the amino acid composition, and is found to be very close to that obtained by electrospray mass spectrometry, 70 990 ± 140 Da.

Data analysis according to an unlimited isodesmic association model (Adams & Lewis, 1968; Adams et al., 1978), in which the equilibrium constants for the addition of monomer to any aggregate are equal, was performed using the SEDPROG software package provided by Greg Ralston [see Ralston and Morris (1993) and references cited therein] and the omega function $\Omega(r)$. $\Omega(r)$ (Milthorpe et al., 1975) is defined as

$$\Omega(r) = \{c(r) \exp[\Phi_1 M_1(r_F^2 - r^2)]\}/c(r_F)$$

where $\Phi_1 = (1 - \bar{v}_1\rho)\omega^2/2RT$, with $\bar{v}_1$ the partial specific volume of the smallest assembling species, ρ the solvent density, ω the angular velocity of the rotor, R the gas constant, and T the absolute temperature of the sedimentation equilibrium experiment. M_1 is the molar mass of the monomer, $c(r)$ and $c(r_F)$ are the total weight concentrations of the associating solute at radial position r and an arbitrarily chosen reference position, r_F, respectively. The parameters of self-association were determined by direct fitting of the omega function to various models according to Morris and Ralston (1985), Ralston and Morris (1992), and Winzor and Wills (1994).

Although thermodynamic nonideality was addressed, the values of the second virial coefficient B returned by the fitting procedure, were close to 0 since they varied from 8 × 10^{-7} to 20 × 10^{-7} L mol/g^2, thus indicating that nonideality is not significant during HSC70 sedimentation.

RESULTS

Analysis of Purified HSC70 by Polyacrylamide Gel Electrophoresis and Chemical Cross-Linking. HSC70 overproduced in *Escherichia coli* has been purified to homogeneity as described previously (Benaroudj et al., 1994). Further analysis of the purified protein, which is in the ADP-bound form, revealed an intrinsic polydispersity. As shown in Figure 1, although the preparation of HSC70 appeared homogeneous on a denaturing gel (Figure 1A), multiple bands corresponding to various oligomeric forms are observed on a nondenaturing gel (Figure 1B). When HSC70 was cross-linked with glutaraldehyde and then submitted to electrophoresis in denaturing conditions, five species, with molecular weights ranging from monomer to pentamer, could be distinguished, and even higher molecular weight species, such as hexamer and heptamer, were discernible (Figure 2, lane 2). These various oligomeric forms appeared even at low protein concentrations (Figure 2, compare lanes 2 and 5), indicating that cross-linking occurred between subunits of stable oligomers rather than between molecules colliding randomly.

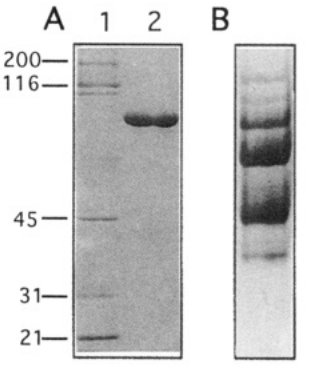

FIGURE 1: Analysis of the purified recombinant HSC70 by polyacrylamide gel electrophoresis (PAGE). (A) SDS−PAGE: lane 1, molecular mass markers (values in kDa on left); lane 2, HSC70 (10 μg). (B) Native PAGE; HSC70 (40 μg).

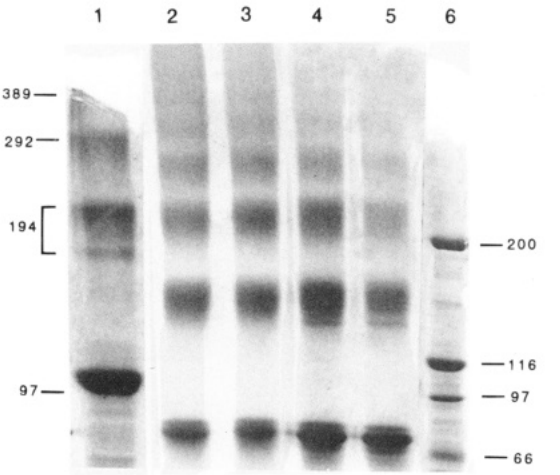

FIGURE 2: Cross-linking of HSC70. Purified HSC70 at a concentration of 3 mg/mL (lane 2), 1 mg/mL (lane 3), 0.3 mg/mL (lane 4), and 0.1 mg/mL (lane 5) was treated with glutaraldehyde (final concentration of 0.1%) as described under Materials and Methods, and then analyzed by SDS−PAGE. Lane 1, cross-linked phosphorylase *b*; lane 6, molecular mass markers (values on the right and the left are in kDa).

Analysis of HSC70 Multiple Species by Size-Exclusion Chromatography. To obtain further information on the nature and the proportion of the multimers present in the HSC70 preparation, the protein was submitted to size-exclusion chromatography. As shown in Figure 3, the protein eluted in two overlapping peaks, a major sharp one corresponding to species having a molecular mass of about 70 kDa and a minor broad one corresponding to species having a molecular mass of approximatively 150−300 kDa. An additional peak corresponding to species having a molecular mass above 440 kDa was observed when the protein was brought to high concentrations (>10 mg/mL) before storage and then analyzed in the same fashion (data not shown), thus confirming that the protein exists as multiple species involving monomers to hexamers and probably higher molecular mass species. In fact, the use of a size-exclusion chromatography column with a wider molecular mass separation range followed by cross-linking of the column fractions showed that species corresponding to hexamers and even higher order oligomers could be detected (Benaroudj et al., 1994). Progressive dilution of the protein leads to a decrease in the proportion of the oligomeric species in favor of that of the monomeric species (Figure 3B,C).

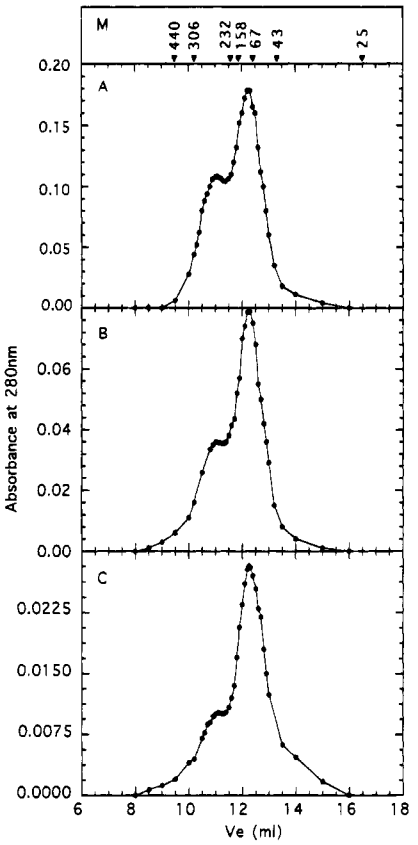

FIGURE 3: Analysis of HSC70 by size-exclusion chromatography. 500 μL of protein at a concentration of 3 mg/mL (A), 1 mg/mL (B), and 0.3 mg/mL (C) was loaded on a Superose 12 column and eluted as described under Materials and Methods. The protein eluted from the column was detected by the UV absorbance at 280 nm (●). *M*, molecular mass standards. These standards were used for the calibration curve representing the Stokes radius as a function of the distribution coefficient K_{av} (Siegel & Monty, 1966; Lemaire et al., 1986). The standards used were ferritin (MW = 440 kDa, R_s = 61Å), catalase (MW= 232 kDa, R_s = 52.2Å), aldolase (MW = 158 kDa, R_s = 48.1Å), albumin (MW = 67 kDa, R_s = 35.5Å), ovalbumin (MW = 43 kDa, R_s = 30.5Å), and chymotrypsinogen A (MW= 25 kDa, R_s = 20.9Å).

Analysis of HSC70 Self-Association Properties by Sedimentation Velocity. Sedimentation velocity confirmed the polydisperse nature of HSC70 by the presence of broad boundaries. A "Schlieren" analysis of these boundaries gives three overlapping peaks, suggesting that the different species present in solution are in a relatively slow exchange so that at least partial separation occurs during the experiment. Since the "Schlieren" analysis involves a numeric differentiation of the data and a certain degree of smoothing that could introduce a bias in the interpretation, raw sedimentation data were directly fitted by nonlinear least-squares procedures as described by Philo (1994). As expected, the data fitted poorly to a one- or two-component system. However, as shown in Figure 4, the data fit relatively well to a three-component model system involving a 4.2S species, a 6.4S species, and an 8.5S species with proportions of about 45%, 35%, and 15%, respectively, at a concentration of HSC70 of 1.6 mg/mL. Nevertheless, some deviation of the fit relative to the experimental data is observed due probably to the fact that faster sedimenting species are present that are not taken into account in the fit, as indicated by the positive slope in the upper plateau of the experimental data relative to the fit, and the large diffusion coefficient (*D*)

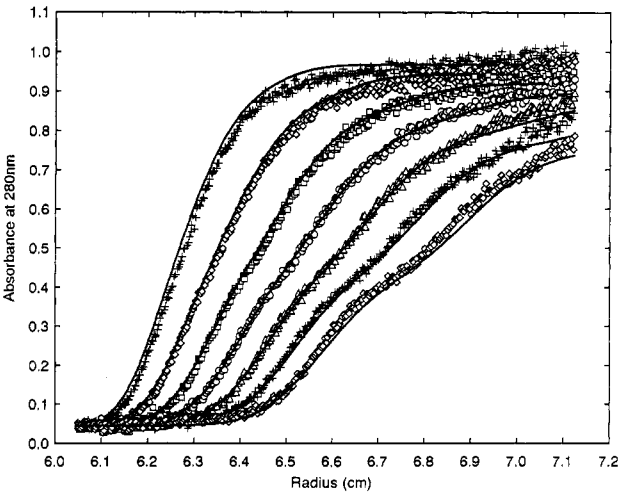

FIGURE 4: Analysis of HSC70 by sedimentation velocity. HSC70 (1.6 mg/mL) was sedimented, and the data, recorded at increasing sedimentation time [36 min (+), 46 min (◇), 56 min (□), 66 min (○), 76 min (△), 86 min (+), and 96 min (◇)], were analyzed as described under Materials and Methods. The fitted data curves are represented by a solid line.

values returned by the fitting procedure. When the data range for each data sets was confined as close to the boundary as possible, in order to minimize the effects of the high molecular weight species, the fit was substantially improved, giving sedimentation coefficient values comparable to those reported above, but still high *D* values that precluded an estimation of molecular weights for the three species from the *s*/*D* ratios. However, using the relation $(s_1/s_2)^3 = (M_1/M_2)^2$ and bovine serum albumin as a reference (Lin et al., 1991), one can obtain apparent molecular masses of about 67, 125, and 182 kDa for the three species, which is compatible with HSC70 monomer, dimer, and trimer. Moreover, the ratio of the sedimentation coefficient of the HSC70 dimer or trimer over that of the monomer is 1.53 and 1.97, respectively, values close to the expected theoretical values of 1.5 and 2 for a dimer and a triangular trimer (Van Holde, 1975).

Plotting the corrected sedimentation coefficients, $s_{20,w}$, obtained either by directly fitting the data as described above or from the "Schlieren" peaks, as a function of protein concentration gives Figure 5A. The $s_{20,w}$ value for each species is independent of HSC70 concentration up to 1.6 mg/mL. Extrapolation to infinite dilution gives $s^0_{20,w}$ values of 4.3, 6.6, and 8.5 S for HSC70 monomer, dimer, and trimer, respectively (Table 1). The frictional ratio values (f/f_0) suggest that all three species are asymmetrical (Table 1).

The variation of the mass fraction of each sedimenting species as a function of HSC70 concentration is shown in Figure 5B. Whereas the proportion of the 4.3S species decreased, those of the 6.6S and 8.5S increased with increasing HSC70 concentration, indicating that HSC70 dimers and trimers are formed at the expense of monomers as expected for a mass-action law equilibrium.

Analysis of HSC70 Self-Association Properties by Sedimentation Equilibrium. Sedimentation equilibrium measurements of HSC70 self-assembly were performed at three initial loading concentrations and three rotor speeds. Least-squares analysis of each dataset for the determination of the weight-average molecular mass is reported in Table 2. For each rotor speed, the weight average molecular mass increased

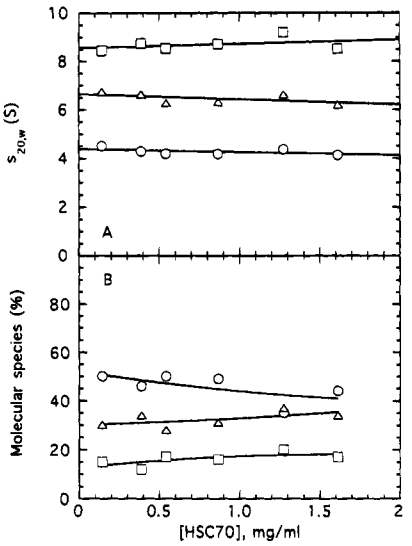

FIGURE 5: Dependence of sedimentation coefficient and relative proportions of the various HSC70 species on protein concentration. $s_{20,w}$ (A) and relative amounts (B) for HSC70 monomer (○), dimer (△), and trimer (□) were determined as described under Materials and Methods using various initial concentrations as indicated on the figure.

Table 1: Hydrodynamic Parameters of the HSC70 Monomer, Dimer, and Trimer[a]

parameter	monomer	dimer	trimer
molecular mass (Da)	70 870	141 740	212 610
$\bar{v}$ (cm^3 g^{-1})	0.735	0.735	0.735
$S^{\circ}_{20,w}$ (S)	4.3 ± 0.1	6.6 ± 0.1	8.5 ± 0.2
f/f_0	1.22	1.28	1.30
R_s (Å)	30.9	38.8	40.4

[a] The conformational parameters were calculated as described under Materials and Methods, using the molecular mass and the partial specific volume values determined from the amino acid composition. $s_{20,w}$ is the sedimentation coefficient; f and f_0 are the frictional coefficients and R_s the Stokes radius.

Table 2: Concentration Dependence of HSC70 Weight-Average Molecular Mass (M_w)[a]

speed (rpm)	HSC70 concn (mg/mL)	M_w (Da)	rms
8000	0.30	129 700 ± 1900	0.006
	0.60	141 000 ± 1700	0.007
	1.20	140 800 ± 1900	0.023
12 000	0.30	111 900 ± 1600	0.006
	0.60	127 100 ± 2000	0.013
	1.20	127 800 ± 2300	0.030
16 000	0.30	114 300 ± 1800	0.014
	0.60	117 600 ± 2100	0.020
	1.20	123 200 ± 3200	0.024

[a] The weight-average molecular mass is obtained assuming no self-association reaction, by fitting the equilibrium sedimentation data to a single ideal species as described under Materials and Methods. The rms is defined as the square root of the variance of the fit and is expressed in optical density units.

slightly, up to 140 kDa, with increasing HSC70 concentration, indicative of self-association. This is confirmed by plotting the variation of the weight-average molecular mass as a function of HSC70 concentration, for a single initial loading concentration (Figure 6), which shows that at the lowest protein concentration the weight-average molecular mass is about the molecular mass of monomer and at the highest protein concentration the weight-average molecular mass approaches the molecular mass of the dimer. However,

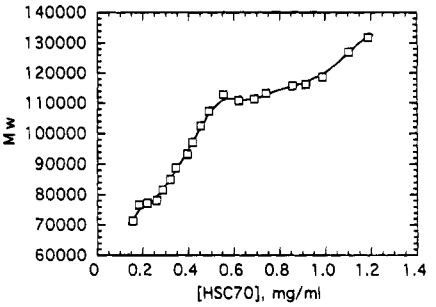

FIGURE 6: Variation of the weight-average molecular mass of HSC70 as a function of protein concentration. HSC70 (1 mg/mL) was centrifuged at 4 °C at 12 000 rpm and analyzed as described under Materials and Methods. The weight average molecular mass (M_w) is calculated from d ln c/dr^2 data, on a point by point basis, using a window of 20 points that moves through the entire data point range. The M_w values are given in daltons, and only selected points (□) are shown.

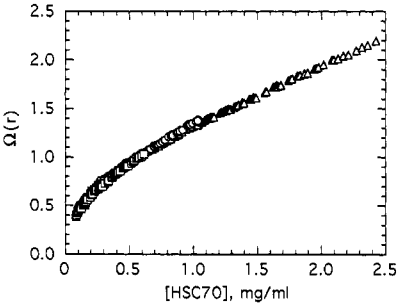

FIGURE 7: Analysis of sedimentation equilibrium of HSC70 by means of the Ω(r) function. Sedimentation equilibrium experiments were performed at 4 °C and at 12 000 rpm as described under Materials and Methods using 0.3 mg/mL (□), 0.6 mg/mL (○), and 1.2 mg/mL (△) HSC70. A reference concentration of 0.3 mg/mL has been used to calculate the Ω(r) values for each set of data. Comparable profiles were obtained for the experiments done at 8000 rpm and 16 000 rpm.

dimers are clearly not the end products of the associative reaction since the weight average molecular mass does not seem to reach a plateau at high concentrations.

A slight dependence of the weight-average molecular mass on rotor speed is observed (Table 2), suggesting that the system might not behave as a perfectly reversible thermodynamic equilibrium. Therefore, and in order to better assess the reversibility of the self-association reaction and to know whether the system has attained chemical equilibrium within the time-scale of the experiment (about 24 h), the data were analyzed using the omega function (see Materials and Methods) according to Milthorpe et al. (1975). This method has been shown to be a sensitive means of demonstrating not only attainment of chemical equilibrium but also the absence of contaminants or materials that do not participate in the equilibrium (Ralston & Morris, 1992). As shown in Figure 7, a nearly perfect superposition of the omega function plots for three loading HSC70 concentrations is observed, indicating that an equilibrium, with the participation of all species, was achieved, and that the self-associating solute behaves as a single thermodynamic component. Altogether, these results suggest that HSC70 self-associates, in a reversible manner, to form dimers, trimers, and larger oligomers with the monomer as the basic assembly unit.

In order to define the mechanism of HSC70 self-assembly and measure the equilibrium constants and the associated thermodynamic parameters, nine datasets, obtained with three

Table 3: Association Constants and Free Energies of HSC70 Self-Assembly[a]

model	association constant	$\Delta G°$ (kcal mol^{-1})	rms
monomer−dimer	$K_{1-2} = 25.9 \times 10^5$ M^{-1}	−8.1	0.014
monomer−trimer	$K_{1-3} = 3.3 \times 10^9$ M^{-2}	−12.0*	0.013
monomer−dimer−	$K_{1-2} = 1.1 \times 10^5$ M^{-1}	−6.3	0.011
trimer	$K_{2-3} = 0.9 \times 10^5$ M^{-1}	−6.2	
	$K_{1-3} = 9.5 \times 10^9$ M^{-2}	−12.6*	
isodesmic	$K = 1.2 \times 10^5$ M^{-1}	−6.4	0.013

[a] Equilibrium sedimentation data were analyzed according to Materials and Methods. Molecular mass was fixed to that of the monomer, calculated from the amino acid composition (70 870 Da). Association constants were calculated by a simultaneous fit of nine datasets to a single set of constants. All the $\Delta G°$ values are given on a "per monomer" basis, i.e., the free energy of adding successive monomers, except for those marked with an asterisk that are given for the trimer. The rms is defined as the square root of the variance of the fit and expressed in optical density units.

initial loading concentrations and three rotor speeds, were simultaneously fitted to a single set of association constants common to all nine experiments. Two classes of models were considered: discrete self-association models with defined stoichiometry and an unlimited isodesmic model. As shown in Table 3, the monomer−dimer−trimer model offers a better fit than the others (monomer−dimer and monomer−trimer models) based on the square root of the variance and the distribution of the residuals (Figure 8). However, the deviation of the residuals at the higher end of the concentration gradient indicates that the degree of oligomerization of the protein higher than the trimer is not adequately taken into account by this model. Interestingly, it appeared that the free energies for adding a monomer to an existing monomer or to a dimer are almost identical (Table 3), suggesting that the data might equally well be fitted by an unlimited isodesmic model, in which the free energies for adding successive monomers would be equal. Indeed, fitting of the data by an isodesmic model gives association constant and free energy values nearly identical to those obtained for the monomer−dimer−trimer model (Table 3), suggesting that HSC70 could self-associates in an unlimited fashion.

Influence of ATP on HSC70 Self-Association. To examine the effect of ATP on HSC70 self-association, the protein preparation was incubated in the presence of ATP and then submitted to size-exclusion chromatography as well as sedimentation velocity. From the chromatographic profiles (Figure 9), it can be seen that, in the presence of ATP, the peak corresponding to the high molecular mass species disappears in favor of a single, symmetrical peak eluting between 67 and 150 kDa, indicating that ATP induces the dissociation of HSC70 self-assemblies. Oligomers do not form, even at high protein concentrations, as long as ATP is always present (N.B. and M.M.L., unpublished data). The same result was obtained by sedimentation velocity. Whereas in the absence of ATP the protein is distributed in 56% monomer (4.3 S), 17% dimer (6.6 S), and 10% trimer (8.5 S), more than 80% of the protein is monomeric in the presence of ATP, giving a sedimentation coefficient of 4.9 S (data not shown).

DISCUSSION

The results described above, obtained using a wide variety of biophysical and biochemical techniques, shed some light on the structure of HSC70 in solution and reveal the mechanism by which the molecular chaperone HSC70 self-associates, thus providing the thermodynamic parameters involved in the assembly process.

Conformational Properties of HSC70. Size-exclusion chromatography and sedimentation velocity show that HSC70 is an asymmetrical molecule having a Stokes radius of 39 Å, larger than expected, and a sedimentation coefficient of 4.3 S, smaller than expected, for a spherical particle of 70 kDa. Even though it can be argued that the self-association properties of the protein might have affected the hydrodynamic parameters of the monomer, comparable values have previously been found for the Stokes radius (Schlossman et al., 1984) and for the sedimentation coefficient obtained on sucrose gradients (Welch & Feramisco, 1985). Furthermore, using these values, a molecular weight of 73 100 could be calculated, in good agreement with the molecular weight of 70 870 calculated from the amino acid composition.

From the sedimentation coefficient determined here and the molecular weight calculated from the amino acid composition, an apparent frictional ratio of 1.22 is obtained, confirming the highly asymmetric nature of the HSC70 monomer. Even though the N-terminal two-thirds of the protein is globular in shape as it appears from the X-ray structure of the ATPase domain (Flaherty et al., 1990), the 3 α-helices of the C-terminal third, made of 20−30 amino acids each, and extending over 80 amino acids residues, according to secondary structure predictions (Sadis et al., 1992; Park et al., 1994; Hightower et al., 1994), could give an overall elongated shape to the molecule. In addition, similar results were found for the bacterial homolog of HSC70, DnaK, which was shown to have an elongated shape by size-exclusion chromatography and quasi-elastic light scattering (Shoenfeld et al., 1995), suggesting that this might be a common feature of the HSP70 protein family.

Self-Association Properties of HSC70. Although previous studies, using polyacrylamide gel electrophoresis and size-exclusion chromatography, indicated that HSC70 existed as a mixture of monomers, dimers, and trimers (Schlossman et al., 1984; Schmid et al., 1985; Guidon & Hightower, 1986; Palleros et al., 1991; Kim et al., 1992), this work shows clearly that trimers are not end products, but rather intermediates in a self-association scheme that goes beyond the formation of trimers. Our previous work using size-exclusion chromatography already suggested the presence of high molecular weight species (Benaroudj et al., 1994), and we show here that the protein could efficiently be cross-linked into various forms going from dimers to hexamers, even at low protein concentrations, thus indicating not only that these high molecular weight species do exist but also that they are not the products of nonspecific interactions. Furthermore, the appearance on the chromatographic profiles of a peak eluting in the dead volume of the column, and corresponding to species of molecular mass higher than 440 kDa, when highly concentrated HSC70 preparations were used (our unpublished data), is also indicative of the presence of oligomers higher than the trimers. Therefore, species higher than trimers need to be considered and included in the self-association scheme of HSC70.

Both size-exclusion chromatography and sedimentation velocity indicated that these multiple HSC70 species are in a rather slowly interconverting chemical equilibrium, and are not mere constituents of a mixture. Indeed, it appears

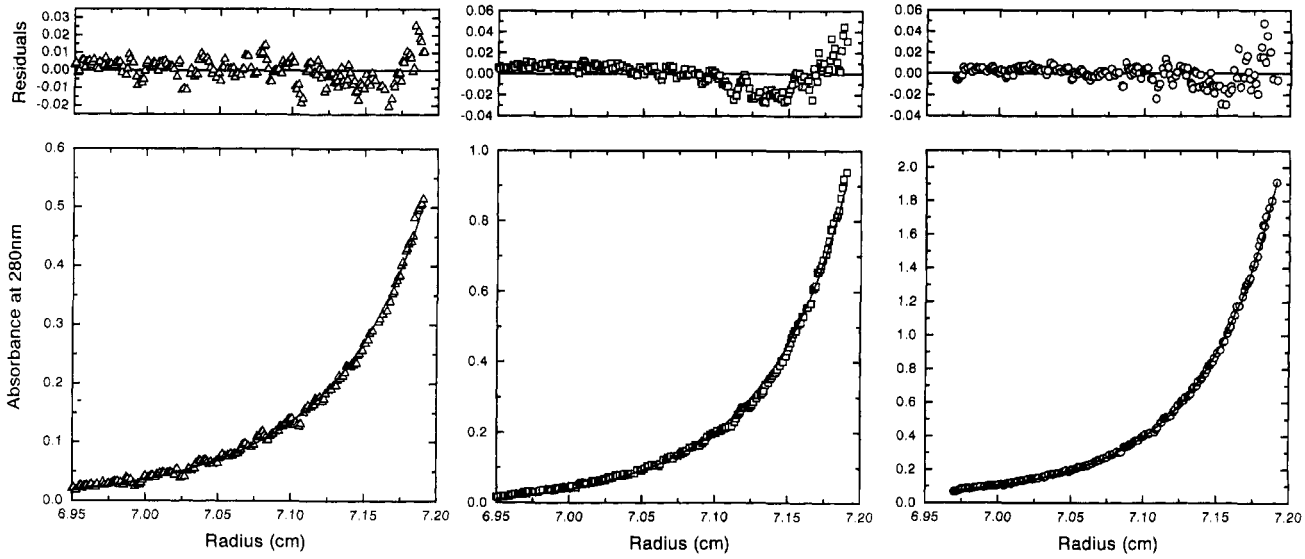

FIGURE 8: Monomer–dimer–trimer fit of the HSC70 equilibrium sedimentation data. Equilibrium sedimentation data, obtained at 4 °C and at 8000, 12 000, and 16 000 rpm using 0.3 mg/mL (△), 0.6 mg/mL (□), and 1.2 mg/mL (○), were simultaneously fitted to a monomer–dimer–trimer model as described under Materials and Methods. The symbols represent the experimental data from the 12 000 rpm run whereas the solid line is the result of the simultaneous fit of nine data sets. The residuals representing the variation between the experimental data and those generated by the fit are also shown (see also Table 3).

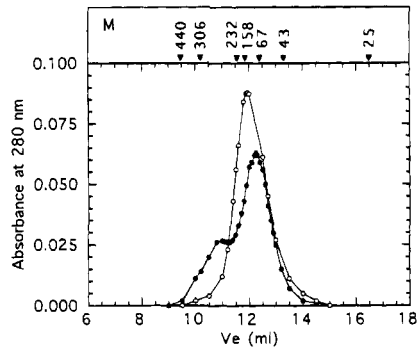

FIGURE 9: Effect of ATP on HSC70 self-association. 500 μL of protein at a concentration of 1 mg/mL in the absence (●) or presence (○) of 100 μM ATP was loaded on a Superose 12 column and eluted as described under Materials and Methods. The protein eluted from the column was detected by the UV absorbance at 280 nm. *M*, molecular mass standards used for calibration as described in Figure 3.

not only that the HSC70 oligomers tend to dissociate upon dilution, but most importantly that the association state of the protein is dependent on protein concentration as expected for a chemical equilibrium. In addition, the fact that each species of the equilibrium could be characterized by a distinct sedimentation coefficient that is not affected by increasing HSC70 concentration, as if it existed in a mixture of several noninteracting species, is indicative of a slowly equilibrating system as compared to the time of sedimentation (about 2 h), and is in agreement with the chromatography results showing a partial separation of the species during the experiment (about 2 h). Though slowly interconverting, the system seems to reach chemical equilibrium within the time range of the equilibrium sedimentation experiment (about 24 h) as indicated by the overlap of the omega functions over the common concentration range. Furthermore, the fact that multiple sets of data, obtained at different initial concentrations as well as various rotor speeds, could be simultaneously fitted to a single set of equilibrium constants is a strong indication of the reversibility of the self-association reaction.

The data fit best to a discrete monomer–dimer–trimer model in which the association constants for adding successive monomers are almost equal and of the order of 10^5 M^{-1}. This result, in conjonction with the other results from gel electrophoresis, cross-linking, and analytical ultracentrifugation indicating the presence of species with higher molecular weights, led us to consider an indefinite, isodesmic assembly model. This model fits the data reasonably well and, most importantly, gives an association constant of the same order as that obtained with the monomer–dimer–trimer model, suggesting that HSC70 might assemble in an isodesmic fashion. Alternatively, it is possible that the protein polymerizes until a stable structure, such as an hexameric ring, for example, is reached. This is quite possible in view of the fact that hexameric ring structures made of a trimer of DnaK and a trimer of DnaJ have been found in thermophilic bacteria (Motohashi et al., 1994).

Role of ATP/ADP in the Self-Assembly Process. It is clear from chromatography and sedimentation velocity studies that ATP induces the dissociation of HSC70 oligomers to monomers. In fact, as long as ATP is present, oligomerization of the protein does not occur. Conversely, once the free ATP is separated from the protein by gel filtration, and the ATP bound has been hydrolyzed, oligomers re-form (Benaroudj et al., 1994). Moreover, there is now evidence that ATP binding, and not hydrolysis, is responsible for the stabilization of the monomeric form (N.B. and M.M.L., unpublished data). Thus, it appears from these studies that the ATP-bound form of the protein is essentially monomeric. Therefore, and since the protein that self-associates is in the ADP-bound form, it is reasonable to conclude that the ATP–ADP couple plays a crucial role in regulating the self-assembly process, ATP shifting the oligomer–monomer equilibrium toward the monomer, and ADP stabilizing the oligomeric forms.

Functional Implications. The tendency of HSC70 to self-associate raises the question of the functional significance of such a structural feature. Since ATP–ADP exchange has been shown to be essential for binding and release of the

unfolded protein substrate (Palleros et al., 1993) and, as discussed above, for controlling the HSC70 oligomer–monomer equilibrium state, it is tempting to speculate that the two mechanisms are coupled. Exchange of ADP for ATP would promote not only the dissociation of oligomers into monomers but also the release of bound substrate. This hypothesis would implicitly mean that the oligomeric form of HSC70 has a higher affinity for the unfolded protein substrates than the monomeric form. Several lines of evidence suggest that this is indeed the case. Heuser and Steer (1989) showed that HSC70 cycles between two allosteric conformations: an ATP-bound state, in which the protein has a low affinity for itself as well as for clathrin, and an ATP-free state, presumably ADP-bound, where both of these affinities are high, and stressed the fact that polymerization of the protein is a prerequisite to tight binding to clathrin and destabilization of the clathrin lattice. Likewise, Wild et al. (1992) provided strong genetic evidence that the functional DnaK in vivo is multimeric by isolating dominant negative mutations, and Blond-Elguindi et al. (1993) showed that biotinylated peptides bind to both monomeric and oligomeric foms of BiP. Moreover, HSC70 has been shown to interact with nascent polypeptide chains (Beckman et al., 1990) in high molecular weight protein complexes (Hansen et al., 1994). Most importantly, on the basis of the fact that extended polypeptide chains interact more strongly with chaperones than do short peptides (Hellebust et al., 1990), Nilson and Anderson (1991) proposed that molecular chaperone of the HSP70 family may acquire high affinity of binding by oligomerization. Altogether these observations stress the importance of HSC70 oligomers in the chaperone function and give support to the hypothesis presented in this report according to which the self-association properties of HSC70 would be coupled to the protein binding properties.

ACKNOWLEDGMENT

We are very grateful to Les Holladay, John Philo, Greg Ralston, and Walter Stafford for making their computer programs available and Borries Demeler, John Philo, Greg Ralston, and Paul Voelker for helpful discussions. We also thank Pierre Kerjan for his help with FPLC and Marie-France Carlier, Jean-Renaud Garel, Guy Hervé, Joel Janin, Marc Lemaire, Dominique Pantaloni, and Jean-Pierre Waller for their comments, suggestions, and continuous support.

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BI951332X