The COOH-terminal Peptide Binding Domain Is Essential for Self-association of the Molecular Chaperone HSC70

(Received for publication, July 16, 1996, and in revised form, December 6, 1996)

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We have previously shown that the molecular chaperone HSC70 self-associates in solution into dimers, trimers, and probably high order oligomers, according to a slow temperature- and concentration-dependent equilibrium that is shifted toward the monomer upon binding of ATP peptides or unfolded proteins. To determine the structural basis of HSC70 self-association, the oligomerization properties of the isolated amino- and carboxyl-terminal domains of this protein have been analyzed by gel electrophoresis, size exclusion chromatography, and analytical ultracentrifugation. Whereas the amino-terminal ATPase domain (residues 1-384) was found to be monomeric in solution even at high concentrations, the carboxyl-terminal peptide binding domain (residues 385-646) exists as a slow temperature- and concentration-dependent equilibrium involving monomers, dimers, and trimers. The association equilibrium constant obtained for this domain alone is on the order of 10^8 M^-1, very close to that determined previously for the entire protein, suggesting that self-association of HSC70 is determined solely by its carboxyl-terminal domain. Furthermore, oligomerization of the isolated carboxyl-terminal peptide binding domain is, like that of the entire protein, reversed by peptide binding, indicating that self-association of the protein may be mediated by the peptide binding site and, as such, should play a role in the regulation of HSC70 chaperone function. A general model for self-association of HSP70 is proposed in which the protein is in equilibrium between two states differing by the conformation of their carboxyl-terminal domain and their self-association properties.

Members of the highly conserved 70-kDa heat shock protein family (HSP70)\(^1\) are involved in several cellular processes such as protein folding, assembly and disassembly of multimeric proteins, protein translocation across membranes, protein degradation, and signal transduction (for reviews see Refs. 1–4). They are thought to act as molecular chaperones by transiently binding hydrophobic regions exposed to the solvent in the non-native conformations of proteins, thereby preventing off-pathway reactions that lead to aggregation (5).

A prominent member of this family, the mammalian, constitutively expressed, 70-kDa heat shock cognate protein (HSC70), has been shown to bind peptides and unfolded proteins (6–8) and to possess refolding activity in the presence of the cochaperone DnaJ (9, 10). HSC70 shows a very weak ATPase activity that can be stimulated 2–5-fold upon binding of peptides, unfolded proteins, clathrin light chains, and chaperones of the DnaJ family (11–14). HSC70 seems to function through cycles of binding and release of polypeptide substrates coupled to binding and hydrolysis of ATP (15), in a mechanism involving co-chaperones of the DnaJ protein family and a newly isolated factor, Hip (16).

HSC70 is made of two domains, an NH\(_2\)-terminal domain of 44 kDa (residues 1–384), which binds and hydrolyzes ATP, and a COOH-terminal domain of about 30 kDa (residues 385–646), which contains the peptide binding site (17, 18). The three-dimensional structure of the NH\(_2\)-terminal ATPase domain has been solved by crystallography (19), and the secondary structure topology of the peptide binding site (residues 385–543) has been determined by NMR methods (20). Recently, the structure of a complex between a seven-residue peptide and the COOH-terminal domain of DnaK, the bacterial HSP70, has been established (21). However, the three-dimensional structure of the entire protein is still unknown.

Self-association is a general and well conserved feature of the HSP70 protein family. BiP (22–24), the constitutive HSC70 (6, 25–30), the heat shock-inducible HSP70 (31), plant HSP70 (32), and DnaK (33), all show self-association properties. Nevertheless, the structural basis and molecular mechanism of such properties have remained undocumented.

To address these questions and define the way self-association of HSC70 may relate to its function, we started the investigation of this process by studying the structure of HSC70 in solution. Using a wide range of biophysical and biochemical techniques, we showed that HSC70 self-associates in solution, in a reversible fashion, into dimers, trimers, and probably high order oligomers, with a dissociation constant of about 5–10 \(\mu\)M (34). Next, we analyzed the effects of the natural substrates on this equilibrium, and showed that whereas in the presence of ADP, HSC70 exists as a slow and temperature-dependent monomer-oligomer equilibrium, in the presence of ATP, peptides, or unfolded proteins, this equilibrium is shifted toward the monomer (35). Most importantly, and since the dissociation process of HSC70 oligomers into monomers appeared to be very similar to that of unfolded protein from HSC70, occurring upon ATP binding but not ATP hydrolysis, we proposed that binding of HSC70 to itself may occur via the peptide binding site and may mimic the binding of HSC70 to unfolded protein substrates (35).

To test this hypothesis, we undertook in the present work the identification of the oligomerization domain and analyzed the self-association properties of two mutants corresponding to the NH\(_2\)- and COOH-terminal domains of HSC70.
EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases were from New England Bio-
labs, and T4 DNA ligase was from Pharmacia Biotech Inc. DNA se-
quencing was accomplished using the T3 sequencing kit from Pharma-
cia. PET 1b vector and Nt II-agarose were purchased from Tebu-
Novagen. FPLC products were from Pharmacia, and all other chemicals
were from Merck.

Construction of the Plasmids Expressing HSC70 NH2-terminal and
COOH-terminal Domains—The plasmid carrying the coding sequence
of HSC70 NH2-terminal domain was designed as follows. Site-directed
mutagenesis was used to introduce an NdeI site at the start codon of
the HSC70 coding sequence that had been previously cloned in the
PstI sites of the pUC119 polylinker. Then a PstI site was introduced to
create a stop codon at codon 385, resulting in the replacement of Asp
and Lys384 by a valine and an asparagine, respectively. The resulting
NdeI-PstI fragment was then cloned between the corresponding sites
of the pNB28 expression vector described previously (30). The final plas-
mid, called pFB5, allowed the expression of the NH2-terminal domain
of HSC70 (residues 1–384) in Escherichia coli.

The HSC70 COOH-terminal domain (385–464) was expressed using
the T7 expression system of E. coli. Site-directed mutagenesis was used
to introduce an NdeI site at codon 385 in the HSC70 coding sequence
of the pFB7 plasmid described previously (30), thus leading to the replace-
ment of codon 384 by a start codon. Then, after removal of the
NdeI-NdeI fragment (codons 1–383), the plasmid was ligated on itself, giving
rise to the pNB28 plasmid. Finally, the NdeI-BamHI fragment of
pNB28 was cloned into the NdeI-BamHI sites of PET1b vector, result-
ing in the NH2-terminal fusion of six histidine residues to the HSC70
COOH-terminal domain (residues 385–464). The integrity of all con-
structions described above was verified by nucleotide sequencing (36).

Protein Expression and Purification—Recombinant HSC70 was ex-
pressed and purified as described previously (30, 34). The recombinant
NH2-terminal domain of HSC70 was expressed and purified as de-
scribed for HSC70 except that the first ion exchange chromatographic
step was replaced by a 60% ammonium sulfate precipitation. After cen-
trifugation, the ammonium sulfate concentration of the supernatant was
adjusted to 80%, and the sample was submitted to centrifugation.
The resulting pellet was resuspended in 20 mM Tris-HCl, pH 7.5, 20 mM
KCl, 1 mM β-mercaptoethanol, and 3 mM MgCl2, dialyzed in the same
buffer, and applied onto an ATP-agarose affinity column as described
previously (30, 34).

The HSC70 (His)6-COOH-terminal domain was expressed and then puri-
fied using His-bind resin (Ni2+-agarose), according to the recom-
mandations of the manufacturer. The eluted protein was subjected to a
thrombin digestion to remove the histidine tail, and any uncleaved
His6-COOH-terminal domain was removed by submitting the whole
sample to a chromatography on an Ni2+-agarose column. After elution,
the protein was concentrated and stored at −80 °C as described previ-
ously (30, 34). The protein concentration was determined by the Lowry
method (55) using bovine serum albumin as a standard, and the protein
concentrations given in the tables and figures are based on the molec-
ular mass of the monomer. Activity of the purified proteins was checked
by the measure of the ATPase activity as well as polypeptide substrate
binding.

Electrophoresis—Polyacrylamide gel electrophoresis (PAGE) under
denaturing conditions (SDS) was carried out in 0.75-mm-thick 12% acryl-
amide slab gel according to Laemmli (37). Gel electrophoresis in
native conditions was performed either on a 6 or 10% acrylamide slab
gel according to Kim et al. (29).

Size Exclusion Chromatography—FPLC chromatography was car-
rried out at room temperature on a Superose 12 column equilibrated
with 20 mM Tris-HCl, pH 7.5, 100 mM KCl, and 1 mM β-mercapto-
ethanol as described previously (34).

Sedimentation Velocity—Sedimentation velocity experiments were
performed at 20 °C on a Beckman Optima XL-A analytical ultracentri-
fuge equipped with a An Ti 60 titanium four-hole rotor with two-
channel 12-mm path length centerpieces as described previously (34).
Data analysis was performed using the computer program SVEDBERG
(38) supplied by John Philo. The apparent molecular mass was deter-
mined as described previously (34), using the relation C0M4 =
M0M4/2 and bovine serum albumin as a reference protein of known
molecular mass and sedimentation coefficient (34, 35, 39).

Sedimentation Equilibrium—Sedimentation equilibrium experi-
ments were carried out at 4 °C using three different loading concentra-
tions and three (for the entire protein) or two (for the isolated frag-
ments) rotor speeds. Radial scans of 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 overlaid successive scans and when
a constant average molecular weight was obtained in plots representing
the average molecular weight versus centrifugation time. Sedimenta-
equilibrium data were analyzed using the appropriate functions by
nonlinear least squares procedures provided in the Beckman Optima
XLA software package.

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

\[ C(r) = \delta + C_{1,0} \exp(\sigma(r^2 - r_b^2)) + \sum_{n=1}^{N} C_{n,0} \exp(Nr^2 - r_b^2) \]  

where \( C(r) \) is the total concentration at radius \( r \), \( \delta \) is the baseline offset, \( C_{1,0} \) is the monomer concentration at the reference radius \( r_0 \), \( N \) is the stoichiometry of the reaction, and \( K_0 \) is the equilibrium association constant. \( \sigma \) is defined as follows,

\[ \sigma = M_1(1 - \varphi_d)\omega^{2/2}RT \]  

where \( M_1 \) is the monomer molecular weight, \( \varphi \) is the partial specific
volume, \( \rho \) is the solvent density, \( \omega \) is the angular velocity of the rotor, \( R \) is the gas constant, and \( T \) is the absolute temperature of the sedimen-
tation equilibrium experiment. The equilibrium constant for the mon-
omer-dimer equilibrium, \( K_{1,2} \), and that for the monomer-trimer, \( K_{1,3} \),
are obtained by the fitting procedure, whereas \( K_{1,2} \) is calculated from these values.

Average weight molecular weights (\( \bar{M}_w \)) were obtained for several
rotor speeds and protein concentrations, by fitting the sedimentation
data to a single species using the equations above. The
variation of \( \bar{M}_w \) as a function of protein concentration from single
runs was determined by calculating the \( M_1 \) from the dually di- 
friction \( (1 - \varphi_d)^{2/2} \)  
RT data, on a point by point basis, using a window of 20 points that
moves through the entire data point range.

Data analysis, according to an unlimited isodesmic association model
(40, 41), in which the equilibrium constants for the addition of monomer
to any oligomer are equal, was performed using the SEDPRESO software
package provided by Greg Ralston (Ref. 42 and references therein) and
the \( \omega \) function of Eq. (43) is defined as follows.

\[ \rho(r) = (\rho_d/r) \exp(\Phi_1/r_0^2 - r^2)) \]  

where \( \Phi_1 = (1 - \varphi_d)\rho^{2/2}RT \) with \( \varphi_d \) representing the partial specific volume of the smallest assembling species, \( \rho \) is the solvent density, \( \omega \) is the angular velocity of the rotor, \( R \) the gas constant, and \( T \) the absolute temperature of the sedimentation equilibrium experiment. \( \Phi_1 \) is the molar mass of the monomer, \( e(r) \) and \( e(r) \) are the total weight concen-
trations of the associating solute at radial position \( r \) and an arbitrarily
chosen reference position, \( r_F \), respectively. The parameters of self-asso-
ciation were determined by direct fitting of the \( \omega \) function to an
isodesmic model according to Morris and Ralston (44), Ralston and
Morris (42), and Winzor and Wells (45).

A monomer molecular mass of 29,046 Da and a partial specific
volume of 0.726 ml/mg at 4 °C, calculated from amino acid composition,
were used for the COOH-terminal domain. For the NH2-terminal
domain and HSC70, monomer molecular masses of 42,002 and 70,870 Da
and partial specific volumes of 0.733 and 0.729 ml/mg at 4 °C were used.
The solvent density was taken as 1.00 g/ml.

RESULTS

Analysis by Polyacrylamide Gel Electrophoresis

Although a purified preparation of HSC70 appears homoge-
neous on a denaturating polyacrylamide gel (Fig. 1A, lane 2), it
presents a polydispersity on a nondenaturating gel, and at least three
species are observed (Fig. 1B, lane 1). By contrast, the NH2-
terminal ATPase domain migrates as a single band
whether in native (Fig. 1B, lane 2) or denaturing conditions
(Fig. 1A, lane 3), suggesting that it exists as a single species.
However, the COOH-terminal domain presents an electrophoretic
behavior similar to that of the entire protein. It exhibits
its multiple bands in native conditions corresponding to at least
three species (Fig. 1B, lane 3), although it shows a single
band in denaturing conditions (Fig. 1A, lane 4), indicative of
self-association.
COOH-terminal Domain Is Essential for HSC70 Self-association

Analysis by Size Exclusion Chromatography

To obtain further information about the nature and the relative distribution of the species present in each protein preparation, HSC70 as well as the isolated domains were analyzed by size exclusion chromatography. While the NH2-terminal domain elutes as a single sharp and symmetrical peak corresponding to a single species having an apparent molecular mass of about 36 kDa (Fig. 2B), the COOH-terminal domain of HSC70 elutes in two overlapping peaks, a major and broad one corresponding to species having an apparent molecular mass of about 200 kDa and a minor one corresponding to species having an apparent molecular mass of about 40 kDa (Fig. 2C), indicating the presence of monomeric and various oligomeric species, probably dimers and trimers. Thus, the COOH-terminal domain alone seems to self-associate in a manner similar to that of the entire protein. The partial separation of these species during chromatography is indicative of the presence of either a mixture or a slow equilibrium between species, comparable with that described for the whole protein (Fig. 2, A and C; Refs. 34 and 35).

To know whether the monomeric and oligomeric species of the COOH-terminal domain exist in a noninterconvertible mixture or in a slow equilibrium, we studied the concentration and temperature dependence of self-association for the isolated domain. As shown in Fig. 3, progressive dilution of the COOH-terminal domain leads to an increase in the amount of the monomeric species at the expense of the oligomeric species (Fig. 3, D, E, and F) in a way similar to that observed for the entire protein (Fig. 3, A, B, and C). These results indicate that the COOH-terminal domain, like the entire HSC70, exists as a slow and concentration-dependent equilibrium between oligomeric and monomeric species (see also Ref. 34). This is confirmed by the temperature dependence of the COOH-terminal domain self-association. In fact, varying the temperature in the gel filtration experiments had two purposes: first, to confirm that the multiple species observed exist as a slow equilibrium and not as a mixture, and second, to know whether the interactions involved in stabilizing the multimeric species are of a comparable nature in the whole protein and its COOH-terminal domain. As shown in Fig. 4, for HSC70 as well as its COOH-terminal domain, increasing the temperature leads to the dissociation of the oligomers into monomers. However, the COOH-terminal domain appears to be more stable at high temperatures than the entire protein, since above 42 °C it is still undergoing dissociation into monomers (Fig. 4, G and H), whereas HSC70 becomes aggregated as indicated by the presence of a single peak eluting with the void volume of the column (Fig. 4, C and D), due presumably to heat-induced denaturation and subsequent aggregation. Even at higher temperatures, up to 60 °C, the COOH-terminal domain does not seem to aggregate and rather elutes as a single chromatographic peak corresponding to monomeric species (data not shown).

It is not clear whether increasing temperatures alter the equilibrium distribution of the species, and thus their respective thermodynamic stability, or the rate of conversion between species, although an effect on the rate has been suggested (46). In the absence of calorimetric studies and thermodynamic parameters, interpretation of these results in terms of the nature of forces contributing to stability could only be speculative. Thus, it could be concluded that whether in the case of the COOH-terminal domain or HSC70, the monomer-oligomer equilibrium is temperature-dependent and that the interactions involved in stabilizing their multimeric species are probably of a similar nature, suggesting that self-association of HSC70 could be accounted for by the COOH-terminal domain.

Analysis by Analytical Ultracentrifugation

Sedimentation Velocity—Results of sedimentation velocity experiments are summarized in Table I. An average sedimentation coefficient, \( s_{20,W} \), of 3.46 S is obtained for the NH2-terminal domain. This value corresponds to an apparent molecular mass of 45,240 Da, close to the molecular mass of the monomer predicted from the amino acid sequence (42,002 Da). Increasing protein concentrations, up to 50 \( \mu \)g, did not significantly affect this value, and the NH2-terminal domain remained monomeric over all of the concentration range (data not shown). Moreover, extrapolating the \( s_{20,W} \) values to 0 gives an \( s_{20,W} \) of 3.24 S, which corresponds to a molecular mass value of 41,108 Da, even closer to that of the predicted one.
By contrast, the average sedimentation coefficients determined for the entire HSC70 and the COOH-terminal domain, 6.67 and 4.25 S, respectively, correspond to molecular masses of 121,300 and 61,700 Da, respectively. These values are higher than those predicted from the amino acid sequence for the NH2-terminal domain (70,870 Da for HSC70 and 29,046 Da for the COOH-terminal domain) and reflect the self-associating nature of these proteins. As described previously, the best fit of the sedimentation velocity data of HSC70 was obtained using a monomer-dimer-trimer model (34). Since the COOH-terminal domain seemed to behave like HSC70 in terms of self-association, a three-component model system has been used to fit the sedimentation velocity data. The $s_{20,w}$ values of 2.91, 4.53, and 6.76 S returned by the fitting procedure correspond to molecular masses of 34,900, 67,800, and 119,900 Da, respectively, and are compatible with a monomer, a dimer, and a trimer. Similar fitting of HSC70 data gave values of 4.63, 7.09, and 9.97 S, which are close to $s_{20,w}$ values of HSC70 monomer, dimer, and trimer published previously (34). The fact that each species of the equilibrium could be separated from the others and characterized by a distinct sedimentation coefficient, as if all species coexisted in a noninterconverting mixture, is indicative of a slowly equilibrating system as compared with the time of sedimentation.

Together, these results are in agreement with the chromatography data and confirm that, while the NH2-terminal domain is monomeric in solution, even at high concentrations, the COOH-terminal domain self-associates, in a manner similar to that observed for the entire protein, giving rise to dimers and trimers in a relatively slow concentration- and temperature-dependent equilibrium.

**Sedimentation Equilibrium**—The weight average molecular weight of the entire protein and the isolated NH2- and COOH-terminal domains at similar concentrations (about 8.5 $\mu$M) were determined by fitting the equilibrium sedimentation data to a single species. Whereas the weight average molecular weight of the NH2-terminal domain was found to be 40,000, close to the molecular mass of the monomer obtained from the amino acid composition (42,002 Da), that of the entire protein (127,000) and that of the COOH-terminal domain (65,000) are much higher than expected (70,870 and 29,046, respectively, suggesting self-association of these proteins (data not shown). This is confirmed by measuring the variation of the weight average molecular weight as a function of protein concentration (Fig. 5).
COOH-terminal Domain Is Essential for HSC70 Self-association

Equilibrium sedimentation data were analyzed as described under “Experimental Procedures.” $K_{m1}$ and $K_{m2}$ are association constants for adding a monomer to a monomer and to a dimer, respectively. $K_{m1}$ is the association constant for the formation of trimer from monomer. Association constants were obtained either by simultaneously fitting nine data sets (for HSC70) and six data sets (for COOH-terminal domain) to a monomer-dimer-trimer self-association model or using the fit function, as previously described (34). The values in parentheses are the root mean squares of the fit and are defined as the square root of the variance of the fit and expressed in optical density units. The equilibrium constants $K_{m1}$ and $K_{m2}$ are obtained by the fitting procedure, whereas $K_{m3}$ is calculated from these values.

**TABLE II**

| Association constant | HSC70 | COOH-terminal domain |
|----------------------|-------|----------------------|
| Monomer-dimer-trimer | $K_{m1}$ | $0.4 \times 10^5$ |
| $K_{m2}$ | $9.5 \times 10^4$ | $24.8 \times 10^5$ |
| $K_{m3}$ | $0.9 \times 10^5$ | $6.2 \times 10^5$ |

**DISCUSSION**

The results of this investigation indicate that all of the self-association properties of HSC70 can be accounted for by the association constant for adding a monomer to a monomer or a monomer to a dimer ($K_{m1}$ and $K_{m2}$, respectively) are on the order of $10^5 \text{ M}^{-1}$ (0.4 $\times 10^5$ and $6.2 \times 10^5 \text{ M}^{-1}$, respectively) for the isolated COOH-terminal domain. These constants are similar to those determined for the entire protein ($1.1 \times 10^5$ and $0.9 \times 10^5 \text{ M}^{-1}$) using the same self-association model. By contrast, the data corresponding to the NH$_2$-terminal domain fit very well to a single ideal species model, giving a molecular mass of 42,000 Da, and significantly less well to a monomer-dimer model, giving a dissociation constant in the millimolar range (data not shown), confirming that this domain does not self-associate in solution. Since we showed previously that the sedimentation equilibrium data for HSC70 could also fit to an unlimited association model involving a single association constant for all steps of about $10^9 \text{ M}^{-1}$ (34), the data for the COOH-terminal domain were fitted to the same model. As shown in Table II, an isodesmic, unlimited association model describes the data almost equally well as indicated by the root mean square of the fit. An association constant of the same order of magnitude ($10^5 \text{ M}^{-1}$) is obtained, thus indicating that the COOH-terminal domain alone is sufficient to account for the thermodynamic properties of the protein as a whole.
COOH-terminal Domain Is Essential for HSC70 Self-association

COOH-terminal domain only, the NH2-terminal domain not taking part in the process. Indeed, association between HSC70 molecules through direct interactions involving their respective NH2-terminal domains can be ruled out in view of the fact that, even at high concentration, the isolated NH2-terminal ATPase domain remains monomeric and behaves as a single ideal species. Although the possibility of self-association involving the COOH-terminal domain of one molecule and the NH2-terminal domain of another by a head to tail mechanism cannot be excluded, this type of association seems unlikely, since the COOH-terminal domain alone is sufficient to account for the self-association properties of the whole protein, not only qualitatively but also quantitatively in terms of association mechanism and equilibrium constants. Both the entire HSC70 and the isolated COOH-terminal domain exist as slow temperature- and concentration-dependent equilibria involving monomeric, dimeric, and trimeric species characterized by dissociation constants of the same order of magnitude, in the micromolar range. Moreover, dissociation of the oligomeric forms of the COOH-terminal domain occurs upon peptide binding in a manner similar to that observed for HSC70 oligomers, suggesting that destabilization of HSC70 oligomers is due to the disruption of contacts between COOH-terminal domains upon peptide binding. Thus, association of the protein to itself seems to occur exclusively via its COOH-terminal part (see Fig. 7).

Although the COOH-terminal domain appears to be necessary and sufficient to account for the self-association properties of the entire protein, the oligomerization site within this domain has not been located. However, because peptides as well as unfolded proteins promote the dissociation of oligomers and stabilize the monomer, presumably by competing with the protein itself for the peptide binding site, self-association of the protein via the peptide binding site appears to be the most straightforward explanation. The peptide binding site of an HSC70 protomer could recognize a target site in another HSC70 protomer, as if this site were a peptide in an extended conformation or an unfolded protein, and lead to self-association (Fig. 7). This is corroborated by the fact that dissociation of HSC70 oligomers occurs after ATP binding but not hydrolysis, just as peptide and unfolded protein substrates bound to HSC70 are released upon ATP binding and not hydrolysis (49). Thus, interactions of the protein with itself should be similar to those of the protein with an unfolded polypeptide substrate.

Although the self-association properties of the protein were previously analyzed by a simple scheme in which HSC70 exists as a slow equilibrium between a monomeric form and an oligomeric form (35), the fact that two monomeric structures, differing in terms of their conformation, were characterized by small angle x-ray scattering (47, 50) and fluorescence (51), suggest that two monomeric states are accessible to the protein. Most importantly, these states correspond either to the ADP-bound form or to the ATP-bound form of the protein, which also are known to differ in terms of their self-association properties, the former being able to oligomerize, whereas the latter is stabilized as a monomer (34, 35). Thus, since the ATP-bound form is stabilized as a monomer, a conformational change appears to be necessary to give rise to an alternative monomeric structure that would be able to self-associate. The question is then whether these two states exist in an equilibrium prior to the binding of nucleotides, the effect of nucleotides being to stabilize one state or the other, or only one monomeric state exists (ADP-bound or nucleotide-free), the other one being induced by ATP binding. We favor the first hypothesis, since it is possible now to obtain, by mutagenesis, monomeric states unable to oligomerize and others that are able to do so in the absence of nucleotides. Based on these considerations, if we assume that these two monomeric structures exist in an equilibrium in the absence of nucleotide, the following model can be proposed. In this model, the protein is seen as a slow equilibrium between two monomeric states, state I and state II, differing by the conformation of the COOH-terminal domain and particularly by that of the peptide binding site (Fig. 7). In state I, this conformation is such that a slow equilibrium between two monomeric structures, differing in terms of their conformation, were characterized by dissociation constants of the same order of magnitude, in the micromolar range. Moreover, dissociation of the oligomeric forms of the COOH-terminal domain occurs upon peptide binding in a manner similar to that observed for HSC70 oligomers, suggesting that destabilization of HSC70 oligomers is due to the disruption of contacts between COOH-terminal domains upon peptide binding. Thus, association of the protein to itself seems to occur exclusively via its COOH-terminal part (see Fig. 7).

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Although the self-association properties of the protein were previously analyzed by a simple scheme in which HSC70 exists as a slow equilibrium between a monomeric form and an oligomeric form (35), the fact that two monomeric structures, differing in terms of their conformation, were characterized by small angle x-ray scattering (47, 50) and fluorescence (51), suggest that two monomeric states are accessible to the protein. Most importantly, these states correspond either to the ADP-bound form or to the ATP-bound form of the protein, which also are known to differ in terms of their self-association properties, the former being able to oligomerize, whereas the latter is stabilized as a monomer (34, 35). Thus, since the ATP-bound form is stabilized as a monomer, a conformational change appears to be necessary to give rise to an alternative monomeric structure that would be able to self-associate. The question is then whether these two states exist in an equilibrium prior to the binding of nucleotides, the effect of nucleotides being to stabilize one state or the other, or only one monomeric state exists (ADP-bound or nucleotide-free), the other one being induced by ATP binding. We favor the first hypothesis, since it is possible now to obtain, by mutagenesis, monomeric states unable to oligomerize and others that are able to do so in the absence of nucleotides. Based on these considerations, if we assume that these two monomeric structures exist in an equilibrium in the absence of nucleotide, the following model can be proposed. In this model, the protein is seen as a slow equilibrium between two monomeric states, state I and state II, differing by the conformation of the COOH-terminal domain and particularly by that of the peptide binding site (Fig. 7). In state I, this conformation is such that a limited region would adopt an extended structure, thereby giving rise to possible interactions with the peptide binding site of another molecule and thus to self-association. These interactions would mimic interactions between HSC70 and target peptides or proteins and result, in a first step, in the dimerization of the molecule. Self-association does not end with the formation of the dimer, however, and could proceed, leading to polymerization and formation of high molecular weight structures (34, 35, 52). By contrast, state II of the protein, in which the COOH-terminal domain as a whole and/or a limited region adopt an alternative, perhaps more defined, conformation, would be more stable as a monomer than state I and would have a tendency to close on itself as a result of favorable contacts between the NH2- and COOH-terminal domains. That significant interactions should exist between the NH2- and COOH-terminal domains has been proposed by Freeman et al. (9), who showed that the COOH-terminal EEVD sequence plays an important role in the regulation of the NH2-terminal ATPase activity. Furthermore, coiled-coil regions both in the NH2- and COOH-terminal domains, predicted by Lupas et al. (53, 54), could mediate interactions between the two domains.

2 B. Fouchaq, N. Benaroudj, and M. M. Ladjimi, unpublished observations.
and in particular a limited region, adopts an alternative, perhaps more defined, conformation that stabilizes the monomer and has the tendency to interact with the peptide binding site, thus preventing self-association. However, when HSC70 has to interact with the peptide binding site, thus preventing self-association. In addition, HSC70 monomer-oligomer equilibrium should be submitted to a tight control by the substrates, nucleotides, peptides, and unfolded proteins, as well as regulatory proteins such as the cochaperones that function in concert with HSC70 within the assisted protein folding machinery. ADP as well as cochaperones that promote the ADP-bound state of HSC70, such as DnaJ, would stabilize state I, thus shifting the equilibrium toward self-association, and ATP, peptides, unfolded proteins, or the cochaperones that stabilize the ATP-bound state of HSC70, such as GrpE, would favor state II, thereby promoting monomerization. Although this seems to be the case in vivo (33–35, 52), further investigation is necessary to unravel the biological implications, if any, of HSC70 self-association.

In conclusion, this scheme reinforces the hypothesis according to which self-association may be a mechanism to regulate the chaperone function of HSC70 (35) and provides a structural basis for this regulation. When HSC70 does not have to "chaperone" another polypeptide chain, its peptide binding site is free but may be protected from becoming bound to unsppecific nucleotides, peptides, and unfolded proteins, as well as regulatory proteins such as the cochaperones that stabilize the ADP and DnaJ homologs stabilize the monomeric state. Bottom, the same scheme as above applies for the structural states and self-association equilibrium of the COOH-terminal domain of HSP70 except that nucleotides and the cochaperones that affect nucleotide binding and hydrolysis are not included due to the absence of the NH2-terminal domain.

Acknowledgments—We are very grateful to Bo Fang for help in this work and to Gérard Batelier for assistance in analytical ultracentrifugation experiments.

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