Domain Structure of the HSC70 Cochaperone, HIP*

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The domain structure of the HSC70-interacting protein (HIP), a 43-kDa cytoplasmic cochaperone involved in the regulation of HSC70 chaperone activity and the maturation of progesterone receptor, has been probed by limited proteolysis and biophysical and biochemical approaches. HIP proteolysis by thrombin and chymotrypsin generates essentially two fragments, an NH2-terminal fragment of 25 kDa (N25) and a COOH-terminal fragment of 18 kDa (C18) that appear to be well folded and stable as indicated by circular dichroism and recombinant expression in Escherichia coli. NH2-terminal amino acid sequencing of the respective fragments indicates that both proteases cleave HIP within a predicted α-helix following the tetratricopeptide repeat (TPR) region, despite their different specificities and the presence of several potential cleavage sites scattered throughout the sequence, thus suggesting that this region is particularly accessible and may constitute a linker between two structural domains. After size exclusion chromatography, N25 and C18 elute as two distinct and homogeneous species having a Stokes radius of 49 and 24 Å, respectively. Equilibrium sedimentation and sedimentation velocity indicate that N25 is a stable dimer, whereas C18 is monomeric in solution, with sedimentation coefficients of 3.2 and 2.3 S and fff values of 1.5 and 1.1 for N25 and C18, respectively, indicating that the N25 is elongated whereas C18 is globular in shape. Both domains are able to bind to the ATPase domain of HSC70 and inhibit rhodanese aggregation. Moreover, their effects appear to be additive when used in combination, suggesting a cooperation of these domains in the full-length protein not only for HSC70 binding but also for chaperone activity. Altogether, these results indicate that HIP is made of two structural and functional domains, an NH2-terminal 25-kDa domain, responsible for the dimerization and the overall asymmetry of the molecule, and a COOH-terminal 18-kDa globular domain, both involved in HSC70 and unfolded protein binding.

Molecular chaperones of the 70-kDa heat shock protein (HSP70)† family play an essential role in protein biogenesis (1–3) through cycles of binding and hydrolysis of ATP coupled to cycles of binding and release of polypeptide substrates (4, 5). During this process, HSP70 switches between two forms, an ADP-bound state and ATP-bound state, depending on the efficiency of ATP hydrolysis and that of ADP to ATP exchange and on the modulation by regulatory proteins called cochaperones. In Escherichia coli HSP70 (DnaK), the cochaperone DnaJ activates the hydrolysis of ATP by DnaK, thereby stabilizing the protein in its high affinity ADP form, while the cochaperone GrpE stimulates the exchange of ADP by ATP, thus helping the protein to switch back to its low affinity ATP form (6–9). In mammals, however, the situation is less clear, and only homologues of the DnaK-ATPase stimulation factor DnaJ have been found in all cell compartments (10), the eukaryotic homologues of the ADP/ATP exchange factor GrpE being restricted to mitochondria and chloroplast, two organelles of prokaryotic origin (11). Current models of the regulation of the mammalian HSP70 ATPase cycle involve at least three factors, HSP40, Bag-1, and HIP (12–17).

HIP, a protein of 368 residues, regulates HSC70 chaperone activity by binding to its NH2-terminal domain and stabilizing the protein in its ADP form, which has a high affinity for the polypeptide substrates (18). It also participates in the maturation of the progesterone receptor by cooperating with HSP70, HSP90, and in the maturation complex (19). High resolution structure of HIP has not been determined, but the polypeptide is assumed to comprise a series of consecutive modules based on sequence alignment criteria and mutagenesis studies (20, 21). On this basis, the protein appears to be composed of an NH2-terminal module (residues 1–15) responsible for the protein oligomerization, a central TPR module (residues 113–214) involved in HSC70 binding followed by a highly charged region (residues 229–271) and a GGMP-rich module (residues 282–310) with unknown function. Although this structural representation may give some insight into the overall organization of the protein and its possible relation to function, it gives little information on the number, the nature, and the actual boundaries of the domains in the folded protein. Since protein domains are usually made of well structured, independently folded units that are linked together by solvent accessible, unstructured regions (22, 23), limited proteolysis has been the classical approach used to define domain organization of a protein (24–26). This approach takes advantage of the fact that mild proteolysis will affect preferentially unstructured accessible regions rather than well structured and compactly folded domains (27–29).

In this report, we describe direct studies of the domain organization of HIP based on limited proteolysis of the native protein as well as structural and functional characterization of the isolated domains.

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† The abbreviations used are: HSP70, 70-kDa heat shock protein; TPR, tetratricopeptide repeat; HIP, HSC70-interacting protein; Bag-1, Bcl-2-associated protein 1; HSP90, 90-kDa heat shock cognate protein; TPR, tetratricopeptide repeat; HIP, HSC70-interacting protein; TPR, tetratricopeptide repeat.
**EXPERIMENTAL PROCEDURES**

All size exclusion chromatography experiments were performed using an AKTA-FPLC system and columns from Amersham Biosciences, Inc.

Thrombin from human plasma and a-cholemyosin from bovine pancreas (type VII 1-chloro-3-tosylamido-7-aminoo-2-heptanone-treated) were purchased from Sigma.

Limited Proteolysis—Recombinant HIP was expressed and purified as described by Velten et al. (32). HIP was submitted to proteolysis by thrombin (10−4 units/µg of HIP/µl) or a-cholemyosin (2 × 10−6 units/µg of HIP/µl) at 37°C in buffer A (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) containing 1 mM β-mercaptoethanol and 2.5 mM CaCl2. At the indicated times, an aliquot of the reaction mixture was removed, treated by phenylmethylsulfonyl fluoride at a final concentration of 1 mM to stop the reaction, and analyzed by 12% SDS-PAGE.

NH2-terminal Sequence Determination of Proteolysis Fragments—Proteolysis fragments (~10 µg each) were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Roche Molecular Biochemicals) using the Transblot cell from Bio-Rad. Proteins corresponding to the bands on the polyvinylidene difluoride membrane were then subjected to NH2-terminal automatic sequence determination using an ECL kit (Amersham Biosciences).

Circular Dichroism—The CD spectra of HIP, N25, and C18 at about 0.5 mg/ml in 20 mM sodium phosphate at pH 8 were acquired with a Jobin-Yvon CD6 spectropolarimeter at room temperature. To optimize the signal/noise ratio, the spectra were recorded on two contiguous regions, one between 182 and 200 nm, using an integration time of 5 s per step, and the second between 200 and 260 using a 1-s integration time. At each step of 0.5°, 0.2° was used in both regions. Each spectrum results from averaging five successive scans. The baseline was acquired under the same conditions.

The CD intensities were converted in terms of differential molar extinction per residue. The protein concentrations were determined from amino acid analyses of the samples, using alanine, or tyrosine or phenylalanine as reference. The normalized spectra were then deconvoluted using a previously described software program (33).

**Purification of N25 and C18 Prepared by HIP Proteolysis**—HIP was subjected to proteolysis by thrombin (57 × 10−6 units/µg of HIP/µl) for 40 min at 37°C, and the reaction was stopped by the addition of phenylmethylsulfonyl fluoride at a final concentration of 1 mM. To separate the two domains, the reaction mixture was injected at room temperature onto a Superdex 200 HR10/30 column precolumn equilibrated in buffer A. Fractions of 0.5 ml were collected at a flow rate of 0.5 ml/min, and those containing either N25 or C18 were separately concentrated by ultrafiltration (YM10 membrane; Amicon) and stored at −80°C.

Protein concentration was determined by the method of Lowry using bovine serum albumin as a standard.

**Expression and Purification of Recombinant Proteins**—Vectors expressing N25 or C18 fused to a histidine tag at the amino-terminal extracellular domain were inserted into the corresponding E. coli reaction-amplified fragments of the rat HIP cDNA into a PET28 plasmid (Novagen). N25 contains residues 1–226, and C18 contains residues 227–368 of rat HIP. The two constructions were controlled by nucleotide sequencing.

Tagged N25 and C18 were expressed and purified by affinity chromatography on a Ni2+-nitriiotriacetic acid column, according to the protocol of the manufacturer (Qiagen). The plasmid expressing the C15 mutant was kindly provided by David Smith and Viravan Prapapanich (31). The corresponding protein was purified as described for HIP (32).

**Electrophoresis**—PAGE in denaturing conditions (SDS) was carried out in 0.75-mm-thick 12% acrylamide slab gels. Gels were run using the Mini-Protein II apparatus from Bio-Rad. Western blot was performed by transferring the proteins, after migration on a 15% acrylamide gel, to a nitrocellulose membrane using the Transblot cell from Bio-Rad. The membrane was then incubated with a monoclonal anti-Myc antibody diluted 1:2000 (Invitrogen). This antibody is conjugated to horseradish peroxidase and therefore has been detected by chemiluminescence using an ECL kit (Amersham Biosciences).

**Size Exclusion Chromatography**—Samples of N25 and C18 were diluted 100-fold to a final concentration of 0.5 µM in buffer A (Tris-HCl, pH 7.5, 20 mM KCl) for 4°C. The protein was then incubated with 0.5 mM of effector (HIP, N25, or C18) or lysozyme as a control. Turbidity of the samples under constant agitation was measured at 320 nm for 20 min at 25°C.

**RESULTS**

**Limited Proteolysis of HIP and Analysis by Western Blot and NH2-terminal Sequencing of the Fragments**

HIP was submitted to a mild proteolysis by thrombin and chymotrypsin for different time periods and subsequently an-
Similarly, thrombin cleaved HIP to give the two major fragments of 36 and 18 kDa apparent molecular masses. Longer incubation times with the protease resulted in further cleavage of the 35-kDa fragment that the former fragments accumulated at the expense of the 36- and 18-kDa fragments, as indicated by the kinetics of cleavage and the fact that the latter. This was corroborated by NH₂-terminal sequencing of the proteolytic fragments by Western blot. Since recombinant HIP contains a Myc epitope at its COOH-terminal extremity, a Western blot has been performed to identify the proteolytic fragments that contain the COOH-terminal extremity. Recombinant HIP (lane 1) was digested with either thrombin (lane 2) or chymotrypsin (lane 3), and then samples were submitted to SDS-PAGE and subsequently revealed by Coomassie Blue or anti-Myc antibody. Molecular mass standards (in kDa) are indicated to the left.

Altogether, these results are summarized in Fig. 2, where the cleavage sites are mapped onto the primary structure and sequence modules of HIP as determined previously by sequence alignment, structural modeling, and mutagenesis studies (20, 21, 32). Thus, both proteases cleave HIP within a highly charged region that is adjacent to the TPR region and predicted to have an α-helical structure, yielding an NH₂-terminal fragment of 226 (thrombin) or 237 (chymotrypsin) residues and a COOH-terminal fragment of 160 (thrombin) or 149 (chymotrypsin) residues. Since the work that follows was performed with thrombin proteolysis fragments, and for clarity, the 35-kDa fragment will be named N25, and the 18-kDa fragment will be named C18 (see also Table I), based on their theoretical molecular mass and their respective positions in the HIP sequence.

**Purification and Recombinant Expression of HIP Proteolysis Fragments**

To obtain large quantities of N25 and C18 fragments of HIP for subsequent purification and analysis, limited proteolysis by thrombin was performed on a preparative scale, and the resultant reaction mixture was subjected to size exclusion chromatography. As shown in Fig. 3, two well defined peaks are obtained, peaks 1 and 2, having apparent molecular masses of about 170 and 30 kDa, respectively, that correspond to the N25 and C18 as shown by SDS-PAGE (Fig. 3, inset). Thus, the two fragments of HIP eluted as two distinct species, having well defined molecular masses and Stokes radii, indicating either that they do not interact in the entire protein or that their interaction is lost after cleavage. Furthermore, the fact that N25 elutes as a 170-kDa protein and C18 as a 30-kDa protein, while their molecular mass deduced from amino acid composition corresponds to 25 and 18 kDa, respectively, indicates that N25 is oligomeric whereas the C18 is rather monomeric.

Since these results suggested that N25 and C18 fragments may correspond to two independent structural domains, recombinant expression in *E. coli* of cDNAs corresponding to residues 1–226 (N25) and 227–385 (C18) of HIP fused to a histidine tail have been performed using pET expression vectors. Significant amounts of the respective proteins have been obtained in a soluble form and purified using affinity chromatography (results not shown). Recombinant N25 and C18 were found to be indistinguishable from their proteolytic counterparts in terms of Stokes radius and the ability to inhibit rhodanese aggregation and to bind to the NH₂-terminal domain of HSC70 (results not shown). Thus, N25 and C18 could be expressed in soluble forms and purified from *E. coli* as independent structural and functional units.

**Characterization of proteolytic fragments**

Recombinant HIP was treated with either thrombin or α-chymotrypsin. The resulting fragments were analyzed by SDS-PAGE and N-terminal sequencing.

| Protease | Apparent massa (kDa) | N-terminal residueb | N-terminal sequence |
|----------|----------------------|---------------------|---------------------|
| Thrombin |                       |                     |                     |
| 35       | 1                    | MDPRKVSE            |                     |
| 30       | NDc                  |                     |                     |
| 20d       | 227                  | AΩKIAEH             |                     |
| Chymotrypsin |           |                     |                     |
| 36       | 1                    | MDPRKVSE            |                     |
| 30       | 1                    | MDPRKVSE            |                     |
| 18       | 238                  | ERKREEEI            |                     |
| 13       | 238                  | ERKREEEI            |                     |

* Estimated from migration by SDS-PAGE.

b The number of the residue in full-length HIP is indicated.

c For clarity, the 35-kDa and 20-kDa fragments used in this study are called N25 and C18 based on their theoretical molecular mass and their position in the HIP sequence.

d ND, not determined.
N25 and C18 proteolysis fragments could not only be produced in *E. coli* as active independent units, but they also show a high content in secondary structure comparable with that found in authentic proteins. As shown in Fig. 4A, the far-UV CD spectra of N25 and C18 fragments are typical of well-structured polypeptides with a large amount of \( \alpha \)-helices (maximum at 191–192 nm; minima at 207–208 and 222 nm). The deconvolution of these spectra fits the experimental data well.

As shown in Table II, the proportions of \( \alpha \)-helix and \( \beta \)-structures of N25 are very close to those of HIP. Moreover, the coincidence between the spectrum of the stoichiometric mixture of N25 and C18 and the sum of the individual spectra of isolated fragments (not shown) is in agreement with the absence of any detectable interaction between the fragments as observed by size exclusion chromatography (Fig. 3). By contrast, and as shown in Fig. 4B, there is a difference between the CD spectrum of HIP and that corresponding to the stoichiometric mixture of N25 and C18. This difference clearly indicates a structural change in the corresponding regions of HIP upon proteolysis, or when the fragments are produced as individual polypeptides. This is corroborated by the fact that the proportion of \( \alpha \)-helices in C18 is significantly smaller than in HIP.

Since the N25 and C18 show the features of correctly folded structural domains in terms of chromatographic behavior, recombinant expression, and secondary structure content, a study of their structural and functional properties was undertaken.
Table II
Secondary structure contents of isolated HIP, N25, C18, or an equimolar mixture of N25 and C18 were determined by circular dichroism experiments as described under “Experimental Procedures.”

|            | HIP | N25 | C18 | N25/C18 |
|------------|-----|-----|-----|---------|
| α-helix (%)| 55  | 53  | 40  | 47      |
| Anti-parallel β-sheet (%)| 6   | 9   | 10  |         |
| Parallel β-sheet (%)   | 3   | 4   | 2   | 1       |
| Turns (%)           | 21  | 20  | 33  | 24      |
| Other (%)           | 21  | 27  | 33  | 24      |
| Total (%)           | 1.00| 1.13| 1.08| 1.00    |
| RMS b              | 0.134| 0.132| 0.159| 0.129  |

*a* Equimolar mixture of N25 and C18 fragments.
*b* Root mean square deviation given by the Varselec fitting procedure (30).

Conformational Properties of HIP Proteolysis Fragments

Size exclusion chromatography indicated that N25 and C18 elute as homogeneous proteins of 49 and 24 Å, respectively (Table III). Although for C18, such a radius was expected, that of N25 is too large for a monomeric protein of 25 kDa. Sedimentation velocity confirmed the monodisperse nature of N25 and C18 as indicated by the decrease of sedimentation coefficients with protein concentration, characteristic of nonassociative particles, and s0 ² of 3.2 and 2.3 S were obtained for these two fragments, respectively (Fig. 5). Based on those values and the experimentally determined molecular mass, determined experimentally (see below), frictional ratios of 1.5 and 1.1 could be calculated for N25 and C18, respectively, emphasizing the asymmetric nature of N25 as compared with the C18, which appears to be rather globular (Table III). When N25 and C18 axial ratios, obtained for equivalent hydrated prolate ellipsoid particles, are compared with that of HIP; it appears that the N25 domain possesses, like the entire protein, an elongated shape, whereas the C18 is spherical (Table III).

Quaternary Structure of HIP Proteolysis Fragments

Sedimentation equilibrium data, performed at different concentrations and rotor speeds, could easily be fitted to a dimer model for N25 and monomer for C18 (Fig. 6, bottom) as indicated by the small variation and the random distribution of the residuals representing the variation between experimental and theoretical monomer (17,966 Da), that of N25 is almost exactly two times that of a theoretical monomer, 51,163 Da compared with 25,582 Da.

Functional Properties of HIP Proteolysis Fragments

Inhibition of Rhodanese Aggregation—As shown in Fig. 7, rhodanese aggregates in a refolding buffer, after being denatured in a guanidinium buffer, as indicated by the increase in light diffusion. This aggregation is reduced by about 50% in the presence of either N25 or C18. Interestingly, this inhibitory effect was greatly increased when these two fragments were used together, becoming comparable with that obtained with the entire protein, suggesting an additive effect (Fig. 7). HIP as well as N25 or C18 activity is specific, since lysozyme, a control protein, had no effect on rhodanese aggregation. Moreover, the additive effect of N25 and C18 could not be obtained when either of these fragments was used with lysozyme, thus indicating that it is not merely due to an increase in protein concentration but truly reflects a cooperation between N25 and C18 to inhibit rhodanese aggregation.

Binding to the ATPase domain of HSC70—HIP has been identified as an HSC70-interacting protein using the two-hybrid system with the NH₂-terminal ATPase domain as a bait (18). These results have been reproduced in vitro in binding experiments involving immobilized proteins (20, 21). When the same experiments are performed here, using immobilized HIP, N25, or C18, the results of Fig. 8 (left) are obtained. It can be seen that HIP as well as its respective N25 and C18 fragments were able to retain the NH₂-terminal domain of HSC70. This domain can be eluted from the column in the presence of ADP but in the absence of any other co-chaperone, and specifically HSP40, suggesting a direct binding of HIP or its respective domains to HSC70.

Discussion

Although a modular organization of HIP has been previously proposed, based on various sequence alignment criteria followed by mutagenesis analysis (18, 20, 21), no studies have
been performed directly on the protein. The work reported here takes advantage of limited proteolysis to probe the actual domain organization of the protein and obtain information on the structural and functional properties of the putative domains.

Limited proteolysis by thrombin and chymotrypsin shows that HIP is cleaved essentially in two fragments of about 25 and 18 kDa that appear to be resistant to further major cleavage. These fragments can be digested further by chymotrypsin, but only marginally and only on the COOH-terminal side, suggesting that they possess a rather compact core and, as expected, more accessible extremities. This was confirmed by the fact that the two proteases cleave only within a narrow region following the tetra-tripeptide repeat (TPR), between residues 220 and 240 of HIP, despite the presence of several potential cleavage sites scattered throughout the sequence and despite their different specificity. Most importantly, protein phosphatase 5, another TPR-containing protein (37) on which the TPR region of HIP has been modeled (32), is cleaved primarily in the same region (i.e. beyond the TPR) by trypsin and subtilisin (38), two proteases that exhibit different specificities from those of thrombin and chymotrypsin. Thus, the major cleavage sites of these four proteases are restricted to a 13-residue segment on the aligned sequences of HIP and protein phosphatase 5 (Fig. 9), although protein phosphatase 5 is a much larger and distinct protein, having only the TPR in common with HIP. Hence, these results strongly suggest that the N25 and C18 fragments represent authentic structural domains of HIP.

Support for this interpretation comes from recombinant expression and biophysical and biochemical characterization of the respective fragments. Indeed, not only these fragments could be expressed in and purified from E. coli as independent folding units, they also exhibit well defined properties in terms of secondary structure content, hydrodynamic parameters, gross conformation, molecular mass, and biological activity. For instance, the sequence of HIP that contains the known remarkable features such as the oligomerization site and the TPR subdomain involved in HSC70 binding (20, 21) corresponds to the NH2-terminal 30 kDa of the protein, whereas the sequence that shows no peculiar characteristics in terms of primary structure, except a GGMP repeat, maps to the COOH-terminal 15 kDa. Interestingly, the same regions that are obtained after limited proteolysis can be expressed in E. coli and exhibit the expected structural and functional properties. The N25 appears to be an elongated dimer, like the entire protein,able to bind HSC70, and the C18 is a globular monomer that appears also to be able to bind HSC70. Thus, a two-domain structure with a linkage between residues 220 and 240 seems to be a plausible model for HIP.

This 220–240 region, following the TPR in HIP and in which the protease cleavage sites are located, has been found to be an α-helix by several secondary structure prediction algorithms. 

FIG. 6. Analysis of N25 and C18 fragments by equilibrium sedimentation. Data were obtained at three loading concentrations and three rotor speeds, resulting in nine data sets for each fragment. Since N25 contains the three tryptophan residues of intact HIP, absorbance was measured at 280 and 230 nm for N25 and C18, respectively. Here are shown the experimental data for N25 (○) at 0.5 mg/ml and 12,000 rpm and C18 (○) at 0.15 mg/ml and 20,000 rpm. Data were fitted to a one-species model (solid line) as described under “Experimental Procedures.” The residuals representing the variation between the experimental data and those generated by the fit are shown in the upper panels.

FIG. 7. Effect of HIP and its N25 and C18 fragments on the aggregation of rhodanese. Rhodanese was denatured and then allowed to refold, at a final concentration of 0.5 μM, alone (○) or in the presence of 1 μM HIP (●), N25 (◇), C18 (▼), N25 and C18 (■), N25 or C18 and lysozyme (line), and lysozyme (□) as a control. Keys for all experiments, including controls, are shown to the right. Absorbance at 320 nm has been normalized to 1, based on the data of rhodanese alone (○) at 20 min. All of the experiments were done in triplicate, and the data points shown represent the mean of the three experiments with their corresponding error bars. AU, absorbance units.

FIG. 8. Analysis of the interaction of HIP and its N25 and C18 fragments with the ATPase domain of HSC70. Purified ATPase domain of HSC70 (N-HSC70) or citrate synthase (control) was incubated with immobilized HIP, N25, or C18 on Ni2+-nitrilotriacetic acid-agarose resin. Incubation was performed in the presence of 0.1 mM ADP for 90 min at 4 °C. After extensive washing with buffer containing ADP, specific elution was performed by the addition of 1 mM ATP (ATP), and then tagged proteins were eluted by the addition of 250 mM imidazole (imid.). Elution fractions were analyzed by SDS-PAGE. The positions of N-HSC70 and citrate synthase are indicated by triangles at the right of the gels.
and three-dimensional molecular modeling (32). In fact, in the TPR crystallographic structures available (39–44), the region following the TPR is folded in an α-helix that packs in part against the TPR domain in a similar arrangement to that of the helices within the TPR domain, suggesting that this region is an integral part of the TPR domain (37, 39, 43). The remainder of this long helix, where the protease-sensitive sites are found, is weakly conserved in terms of sequence and more accessible to the solvent and thus could provide a link between the TPR and other domains in proteins. Furthermore, although regions connecting domains in proteins are generally believed to be solvent-accessible, flexible loops, helices are often found in crystallographic structures to be domain linkers. This is in fact the case of the HSP70 COOH-terminal domain, which is composed of a β-sandwich subdomain and a helical bundle subdomain, connected together by a long helix, in the middle of which protease-sensitive sites are found (45). A structural reorganization or “melting” in solution of such regions from a well defined secondary structure to a flexible loop has been proposed to rationalize this observation (45, 46). Together, these data suggest that the α-helix following the TPR may constitute a linker between domains whether in HIP or other TPR-containing proteins.

In view of the fact that the N25 domain appears to be responsible for the dimerization and the elongated shape of the whole protein, since the C18 domain is a globular monomer, and that the first 14 residues are necessary for oligomerization (21), it is tempting to speculate on the association mode of the monomers in the dimer. In principle, the two monomers of HIP can associate in different fashions either through the NH$_2$-terminal regions. As far as functional properties are concerned, it was interesting to find that the C18 domain of HIP is able to bind HSC70. This result indicates that although it does not have a TPR, the C18 participates in HSC70 binding, probably by stabilizing the primary interaction between HSC70 and the N25 domain of HIP, and suggests that, if the TPR is necessary for HSC70 binding, it may not be sufficient. This complementarity between the N25 and C18 domains of HIP in HSC70 binding is also seen in the unfolded rhodanese binding (to inhibit rhodanese aggregation), making it possible that unfolded protein binding and HSC70 binding are mediated by the same site on HIP involving an unidentified region on the C18 domain and the TPR region on the N25 domain. This is supported by the fact that TPRs are not only known to be involved in protein-protein interactions but have also been shown to bind relatively hydrophobic peptides in an extended conformation (37, 39).

On the basis of these hypotheses, a structural scheme for the cochaperone HIP, taking into account the hydrodynamic data and functional properties described in this paper, is presented in Fig. 10. HIP is presented as a dimeric protein in which the two monomers, each one composed of two relatively globular domains, interact through their NH$_2$-terminal extremity, thus giving an elongated shape to the protein. This structure provides two binding sites for HSC70 located in the TPR of the NH$_2$-terminal 25-kDa domain as suggested previously (32) and confirmed in this work. However, the TPR by itself does not seem to be sufficient for efficient binding, and participation of the C18 domain in the formation of the binding site is proposed, since this domain shows HSC70 and unfolded rhodanese binding activities. Therefore, the HSC70 binding site on HIP, that could overlap the unfolded protein binding site, is presented as shared between the two domains.

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FIG. 9. Comparison of proteolysis sites on aligned sequences of HIP and protein phosphatase 5. The sequences of HIP and protein phosphatase 5 (PP5), corresponding to the TPR region and the following helix, have been aligned (see also Ref. 32). Identical residues (line) and conservative replacements (dotted line) are shown. Here are shown the cleavage sites in HIP (above), determined in the present work, and those determined in protein phosphatase 5 (38) (below). Only primary cleavage sites in HIP (HIP) are shown. Here are shown the conservative replacements (conservative replacements) and line helix, have been aligned (see also Ref. 32). Identical residues (identical residues) and conservative replacements (conservative replacements) are shown. Here are shown the cleavage sites in HIP (above), determined in the present work, and those determined in protein phosphatase 5 (38) (below). Only primary cleavage sites are shown.

FIG. 10. Structural model of HIP. This model takes into account the hydrodynamic, limited proteolysis, and functional data described in this paper and by Velten et al. (32). HIP, represented as an elongated molecule of axial ratio a/b of 7.5, is composed of two relatively globular monomers interacting through their NH$_2$ termini. Each monomer is composed of two globular domains, a 25-kDa NH$_2$-terminal domain (N25, large sphere) and an 18 kDa COOH-terminal domain (C18, small sphere), linked together via residues 220–240 (not shown). The dimeric N25 is ellipsoidal like HIP, as indicated by its a/b ratio of 6, while the monomeric C18 is globular (a/b of ~1). This structure provides two potential binding sites for HSC70 (indicated by arrows) located at the interface between the TPR of the 25-kDa domain and an unidentified region of the 18-kDa domain. The HSC70 binding site on HIP may overlap the unfolded protein binding site as suggested by data of Fig. 7.
