Small heat-shock proteins (Hsps) are ubiquitous molecular chaperones which prevent the unspecific aggregation of non-native proteins. For Hsp26, a cytosolic sHsp from of *Saccharomyces cerevisiae*, it has been shown that, at elevated temperatures, the 24 subunit complex dissociates into dimers. This dissociation is required for the efficient interaction with non-native proteins. Deletion analysis of the protein showed that the N-terminal half of Hsp26 (amino acid residues 1–95) is required for the assembly of the oligomer. Limited proteolysis in combination with mass spectrometry suggested that this region can be divided in two parts, an N-terminal segment including amino acid residues 1–30 and a second part ranging from residues 31–95. To analyze the structure and function of the N-terminal part of Hsp26 we created a deletion mutant lacking amino acid residues 1–30. We show that the oligomeric state and the structure, as determined by size exclusion chromatography and electron microscopy, corresponds to that of the Hsp26 wild-type protein. Furthermore, this truncated version of Hsp26 is active as a chaperone. However, in contrast to full length Hsp26, the truncated version dissociates at lower temperatures and complexes with non-native proteins are less stable than those found with wild-type Hsp26. Our results suggest that the N-terminal segment of Hsp26 is involved in both, oligomerization and chaperone function and that the second part of the N-terminal region (amino acid residues 31–95) is essential for both functions.

Introduction

The common trait of small heat shock proteins (sHsps) is the conserved C-terminal domain, the so-called α-crystallin domain. This term refers to the most prominent family member, the eye-lens protein α-crystallin. Apart from the α-crystallin domain, sHsps show heterogeneity in sequence and size. They have been termed “junior chaperones” due to their subunit weight. However, all sHsps investigated up to now form large oligomeric complexes, mainly of 12–42 subunits. Structural analysis of several members of the family showed that they form hollow particles with openings, where the basic building block of the oligomer is a dimer. Several species-specific variations of this common scheme have been described. For example, wheat Hsp16.9 assembles into a dodecameric double disk, each disk organized as a trimer of dimers. There also seem to be critical variations in the stability of the oligomers. While the archaeal Hsp16.5 has a rigid and well-defined quaternary structure, subunit exchange and high flexibility of the oligomeric assembly were detected for α-crystallin and other sHsps. sHsps have been included in the class of molecular chaperones because they bind specifically to unfolded proteins *in vitro* and prevent their aggregation. Compared to other chaperones, sHsp have a remarkable binding capacity of up to one target protein per sHsp subunit. In this context, a striking feature of sHsp is the formation of large, stable complexes of globular shape upon substrate binding.
Hsp26, a member of the family of sHsps in the cytosol of *Saccharomyces cerevisiae*, forms large oligomeric complexes of 24 subunits and suppresses the aggregation of target proteins.\(^6\)\(^{,20,21}\) Interestingly, Hsp26 disassembles into dimers under heat shock conditions, as a pre-requisite for chaperone activity.\(^9\) In addition to the sophisticated regulation of Hsp26 expression,\(^22–24\) this seems to be a second regulatory mechanism of Hsp26.\(^25\)

Hsp26 consists of a C-terminal \(\alpha\)-crystallin domain and, compared to other sHsps, a large N-terminal region. Studies on the isolated \(\alpha\)-crystallin domain of Hsp26 lacking the entire N-terminal region revealed that these regions are essential both for the assembly of the 24mer from dimers and for the interaction with non-native proteins including the formation of the Hsp26–substrate complex.\(^25\) In general, the contribution of the N-terminal, heterogeneous part of sHsps to their structure and function is far from clear.

Here, we analyzed the structural and functional influences of the truncation of the first N-terminal part of Hsp26. We show that the truncated protein is able to form oligomers with 24 subunits. This oligomer is functional as a chaperone and activated by temperature-dependent dissociation. In contrast to full length Hsp26, however, the ability to suppress protein aggregation and the stability of the Hsp26\(\Delta N30\) mutant is decreased. In addition, the N-terminally truncated version of Hsp26 is not able to form stable complexes with unfolded proteins.

**Results**

**Structural analysis of Hsp26\(\Delta N30\)**

According to sequence alignments of several sHsps and hydropathy plots (Figure 1, and data not shown), the N-terminal region of Hsp26 might consist of two parts. In addition, short time partial proteolytic digestion of Hsp26 with proteinase K resulted in two major truncation products (Figure 1A). MS-analysis of the products revealed that both arise from N-terminal truncation and that their molecular masses correspond to species lacking either the N-terminal 21 or 29 amino acid residues (Figure 1A and B).

To determine whether the roles of the N-terminal...
region could be dissected, we analyzed the influence of the truncation of the first 30 N-terminal amino acid residues on the structure and function of Hsp26. We cloned and expressed the truncated protein as described in Materials and Methods. CD spectroscopy demonstrated that the truncated version displayed a spectrum dominated by a high β-sheet content (data not shown). Calculation of the structural elements in comparison to the wild-type protein showed a decrease in the random coil and the α-helical segments (4% and 3%, respectively). The β-sheet content on the other hand was slightly increased (~5%). These results indicate, that the first 30 N-terminal amino acid residues may be incorporated in a α-helical structure, since the α-crystallin domain of sHsps has a high β-sheet content.25

In size exclusion chromatography (SEC) experiments we determined the quaternary structure of the truncated version of Hsp26. Surprisingly, Hsp26ΔN30 still formed oligomers eluting at about the same position as wild-type Hsp26 (Figure 2A). Thus, we conclude that Hsp26ΔN30 is in the same oligomeric state as full length Hsp26.

Next, we investigated whether these oligomers dissociated in a temperature-dependent manner. Like wild-type Hsp26, Hsp26ΔN30 dissociated into a smaller species at 43 °C as analyzed by SEC. This new, smaller species corresponded to a dimer (Figure 2A and B, and data not shown). Furthermore, when the protein was cooled down to 25 °C, it re-assembled into 24mers (Figure 2B).

To investigate differences in the structural assembly of the 24mer in more detail, we studied the complex by electron microscopy (EM) (Figure 3). Negative-stain EM of Hsp26ΔN30 revealed oligomeric particles that appear very similar to those of the full-length protein. A data set of ~2000 particles was aligned and sorted into similar subsets to produce class averages. Like the full-length protein, the Δ30 protein forms particles of two slightly different sizes. These were identified and separated as described.26 For comparison, an equivalent data set of full-length particles was collected and processed in the same way. Example class averages and the sum of all particles in each group are shown in Figure 3. The paired images demonstrate that the truncated and full-length proteins produce very similar assemblies, although the truncated assemblies were more unstable than the wild-type protein. A detailed analysis of the wild-type structure and the origin of the two size groups will be presented elsewhere (H. E. White et al., unpublished results).

Figure 2. Quaternary structure of Hsp26ΔN30. A, Size exclusion HPLC was performed using a TosoHaas TSK 4000 PW column as described in Materials and Methods at 25 °C. Continuous line, Hsp26ΔN30; dotted line, Hsp26. B, Size exclusion chromatography was performed using a TosoHaas TSK 4000 PW column as described in Materials and Methods at different temperatures. Continuous line, Hsp26ΔN30 separated at 25 °C; broken line, Hsp26ΔN30 separated at 43 °C; dotted line, Hsp26ΔN30 incubated at 43 °C for 30 minutes, separated on a pre-equilibrated column at 25 °C.

Figure 3. Electron micrographs of negatively stained Hsp26 and Hsp26ΔN30. For each sample, the two slightly different size groups are shown (A, big assembly; B, small assembly). Four class averages are selected out of a total of 100, and equivalent classes are paired, to illustrate that the N-terminally truncated form produces an assembly that is closely related in size and structure to the full-length protein. The last image in each row is the sum of all centered images in each group. The classes contain about 20 raw images each. The particle diameter is around 200 Å.

Analysis of the N-terminal Domains of Hsp26

447
Having established that the Hsp26ΔN30 oligomer assembly is surprisingly similar to that of the wild-type protein, we investigated the stability of the quaternary structure. In a set of SEC experiments, performed at temperatures varying from 25 °C to 43 °C (Figure 4), the direct comparison of the dissociation behavior of Hsp26 and Hsp26ΔN30 demonstrated that the dissociation of Hsp36ΔN30 starts at 29 °C and is completed at 37 °C. In contrast, Hsp26 starts to dissociate at 35 °C and dissociation is completed at 43 °C. Thus, the quaternary structure of Hsp26ΔN30 is destabilized compared to the wild-type protein.

To analyze if the destabilization is limited to the dissociation of the 24mer, we performed thermal unfolding experiments. Like Hsp26, Hsp26ΔN30 showed two temperature-induced structural transitions, one started at 18 °C with a midpoint at 31 °C (Figure 5). The second transition was between 70 °C and 80 °C with a midpoint at 77 °C corresponding to the complete unfolding of the protein (Figure 5A, and data not shown). Compared to Hsp26, which has a midpoint of the first thermal transition at 35 °C, the thermal stability of Hsp26ΔN30 is slightly decreased. The second transition is identical in both proteins. Similar to wild-type Hsp26, thermal unfolding of Hsp26ΔN30 is completely irreversible.

In an additional experiment, we analyzed the stability of Hsp26ΔN30 against increasing concentrations of urea by CD spectroscopy. Hsp26ΔN30 showed two transitions at 25 °C. The first one

![Figure 4.](image)

*Figure 4.* Comparison of the stability of the quaternary structure of Hsp26 and Hsp26ΔN30. Size exclusion chromatography was performed using a TosohHaas TSK 4000 PW column as described in Materials and Methods at the temperatures indicated. The proteins (Hsp26, left panel; Hsp26ΔN30, right panel) were incubated at the respective temperatures for 30 minutes and separated on a pre-equilibrated column at the respective temperature.
exhibited a midpoint at 0.4 M urea and the second transition was from 3 M urea to 6 M urea and had a midpoint at 4.3 M urea (Figure 5B). In contrast to the heat-induced unfolding, the urea-induced unfolding was completely reversible. A quantitative analysis of the corresponding stabilization energies resulted in values of 4.1 (±1.2) kJ/mol for the first and 17.1 (±1.7) kJ/mol for the second transition. Thus, the first transition indicates a decrease in the stability of Hsp26DN30 compared to Hsp26, whereas the second transition is in agreement with the urea-induced unfolding of wild-type Hsp26.25

Finally, we determined the stability of Hsp26DN30 at 43°C where the protein is in its dimeric form (Figure 5B). Here, the transition started at 2 M urea and was completed at 7 M urea. Again, the transition was reversible and the stabilization energy of the observed transition was calculated as 16.7 (±1.8) kJ/mol corresponding to the unfolding of Hsp26 and Hsp26ΔN at 43°C.20

Functional analysis of Hsp26ΔN30

Next, we set out to analyze the functional properties of Hsp26ΔN30. Previously, we demonstrated that Hsp26 prevents the aggregation of citrate synthase (CS) and other substrates at higher temperatures and forms stable complexes with these proteins,6,20 while a truncated version of Hsp26 corresponding to the α-crystallin domain with the C-terminal tail displayed no chaperone activity.25 With identical ratios of substrate protein to sHsp, Hsp26ΔN30 was slightly less active than Hsp26 in suppressing the aggregation of CS and rhodanese (Rho) (Figure 6). To further investigate this difference in chaperone activity, we analyzed the influence of Hsp26ΔN30 on the inactivation of CS. Hsp26ΔN30 was able to slow down the heat-induced inactivation of CS in a concentration-dependent manner (Figure 6C). Here, compared to Hsp26, lower amounts of Hsp26ΔN30 were sufficient to decelerate the inactivation of CS (Figure 6C).

In additional experiments we analyzed the complex formation of Hsp26ΔN30 with unfolding proteins. Hsp26ΔN30 was incubated with CS at 43°C for 30 minutes, the mixture was cooled down and then applied to SEC at 25°C. Surprisingly, no complexes between Hsp26ΔN30 and CS were detected (Figure 7). When Rho was incubated with Hsp26ΔN30 at 43°C for 30 minutes and the samples were cooled down, the SEC analysis revealed the presence of complexes. But, in contrast to experiments with wild-type Hsp2625, the amount of complex observed was only ~10% of that expected (Figure 7B). In previous experiments, we had shown that complexes of Hsp26 with its substrates are intrinsically more stable than the Hsp26 oligomer.25 To further analyze the chaperone properties of Hsp26ΔN30, we determined the stability of the complexes between Hsp26ΔN30 and Rho in comparison with Hsp26ΔN30. Like the Hsp26 24mer, the Hsp26ΔN30 24mer dissociated at low urea concentrations and dissociation was complete at urea concentrations above 0.3 M (Figure 7). Similar to Hsp26, complexes of Hsp26ΔN30 and Rho were more stable than the Hsp26 oligomers. However, compared to the stability of Hsp26–Rho complexes, where 50% of the complex was still observed at 2.8 M urea, Hsp26ΔN30–rhodanese complexes were less stable (Figure 7).25 Here, 50% of the Hsp26ΔN30–Rho complex were dissociated at concentrations of 1.2 M urea and dissociation was complete at concentrations above 2 M urea.

Discussion

Structural analysis of sHsps revealed that α-crystallin and other sHsps are dynamic oligomers.4,7,9,12,27–30
Two different overall structural assemblies have been described so far: a globular shell-like structure was observed for \( \alpha \)-crystallin, Hsp16.5 from *Methanococcus jannaschii* and Hsp26 from yeast \(^4\) or a double ring like structure for Hsp16.9 from wheat \(^7\) and Hsp42 from yeast. \(^{30}\)

A key feature of Hsp26 is the existence of two distinct, well-defined oligomeric states. The globular shell-like 24mer present at physiological temperatures is largely inactive as a chaperone. The dimer is the stable structure at higher temperature. The inter-conversion between the two species is completely reversible. \(^6\) The isolated \( \alpha \)-crystallin domain of Hsp26 is a stable folded dimer. \(^{25}\) This strongly argues for an important contribution of the N-terminal regions to the stable association of the 24mer. Furthermore, the N-terminal deletion is inactive as a chaperone.

Compared to Hsp16.5 from *M. jannaschii* and wheat Hsp16.9, the N-terminal region of Hsp26 is twice as long. In addition, the N termini of different sHsps share little sequence homologies, \(^31\) limiting a direct comparison of structural data. Analysis of the hydropathy and sequence comparison of different sHsps indicates that in the case of Hsp26, the N-terminal segment might contain two structurally distinct parts. Here, we determined the structural and functional consequences of the deletion of the N-terminal 30 amino acid residues of Hsp26, which correspond to the first part of the N-terminal region of Hsp26. To exclude that a specific secondary structural element was split by the truncation we also tried to express and analyze versions of Hsp26 where five to 35 of the N-terminal residues were truncated in five amino acid residue steps, but with the exception of Hsp26\(_{\text{DN30}}\) they all seemed to be unstable \(\textit{in vivo}\) (data not shown). Surprisingly, the truncation of the first 30 residues resulted in a stable version of Hsp26, which was still able to form 24mers. EM analysis revealed structural features of Hsp26\(_{\text{DN30}}\), which are similar to those of the wild-type protein. Thus, the deletion of the first part of the N-terminal region does not prevent the association to a shell-like structure. However, the stability of this 24mer is decreased, suggesting a contribution of the N-terminal amino acid residues to the association of the subunits in the complex. Compared to wild-type Hsp26, the N-terminal-truncated version dissociated at lower temperatures and less energy was needed for complete dissociation. This demonstrates that the N-terminal segment has an impact on the stability and the dynamics of the 24mer, as its truncation shifts the equilibrium between dimer and oligomer in the direction of the dimer.

This is in agreement with the available structural information on sHsps. The crystal structure of wheat Hsp16.9 shows that one monomer in each and measuring the remaining CS activity, as described in Materials and Methods, at the time points indicated.

**Figure 6.** Influences of Hsp26\(_{\text{DN30}}\) on aggregation and inactivation of substrate proteins. A, Influence of Hsp26\(_{\text{DN30}}\) on the thermal aggregation of CS. CS (final concentration, 3 \(\mu\)M) was diluted into a thermostated solution (43 \(^\circ\)C) of 3 \(\mu\)M (○), 6 \(\mu\)M (■), 12 \(\mu\)M (□) of Hsp26\(_{\text{DN30}}\) and 6 \(\mu\)M Hsp26 (▼). Filled circles (●) represent the spontaneous aggregation of CS at 43 \(^\circ\)C. The kinetics of aggregation were determined by measuring the light scattering of the samples at 400 nm. Concentrations refer to monomers. B, Influence of Hsp26\(_{\text{DN30}}\) on the thermal aggregation of Rho. Rho (final concentration, 1 \(\mu\)M monomer) was diluted into a thermostated solution (44 \(^\circ\)C) of 1.5 \(\mu\)M Hsp26\(_{\text{DN30}}\) (○) and 1.5 \(\mu\)M Hsp26 (▼) monomer. Filled circles (●) represent the spontaneous aggregation of Rho at 43 \(^\circ\)C. The kinetics of aggregation were determined by measuring the light scattering of the samples at 400 nm. C, Influence of Hsp26\(_{\text{DN30}}\) on the inactivation of CS at elevated temperatures. Inactivation of CS (300 nM monomer) at 43 \(^\circ\)C in the absence (●) and in the presence of 3.6 \(\mu\)M (□) and 7.2 \(\mu\)M (▲) Hsp26\(_{\text{DN30}}\) (monomer). For comparison, inactivation of CS in the presence of 7.2 \(\mu\)M Hsp26 (▼) was measured. Inactivation was monitored by incubating the sample at 43 \(^\circ\)C

Analysis of the N-terminal Domains of Hsp26
dimer exhibits a structured N-terminal segment while the other has an unstructured N-terminal part. These N termini seem to be a major feature stabilizing the Hsp16.9 oligomer. In the oligomeric disk the N-terminal arms of two subunits, one of each ring, intertwine in pair-wise fashion creating a knot structure. The three knots formed by this interaction link the oligomer together by hydrophobic contacts. In the crystal structure of M. jannaschi Hsp16.5, the N-terminal arms are unresolved, preventing a detailed analysis of their role in the structure of the oligomer. Our results suggest that in Hsp26 the function of the N-terminal half of the protein can be further dissected. In comparison, Hsp26ΔN, a construct lacking the entire N-terminal part (amino acid residues 1–95), is a stable dimer indicating that the α-crystallin domain and the C-terminal extension alone are not sufficient for the assembly of the oligomer. Thus, the middle region (N-terminal part 2; amino acid residues 31–82) seems to play a substantial role in the assembly of the oligomer and the N-terminal segment modulates this property.

In the crystal structures of M. jannaschi Hsp16.5 and wheat Hsp16.9, C-terminal extensions following the α-crystallin domain are involved in stabilizing the oligomer. The comparison of both assemblies indicates that, while the contact site of the C terminus on a neighboring α-crystallin domain seems to be conserved, the orientation of the C termini is flexible and thus the contacts formed in different sHsps oligomers might vary. Recently it was shown that a basic x-I/V-x I/V motif in the C-terminal extension forms contact with a hydrophobic patch in the α-crystallin domain and that this interaction is critical for oligomer formation. Interestingly, in the case of Hsp26, the C-terminal extension is relatively long and the hydrophobic patch in the α-crystallin domain is missing. The data presented here indicate that in Hsp26 the contact site for the C-terminal extension might reside in the N-terminal region. This view is supported by data from Giese & Vierling who were able to complement mutations in the C-terminal extension with various point mutations in the N-terminal part of Hsp16.6 from Synchocystis. This might be due to the stabilization of a site involved in oligomerization.

sHsps are not only able to suppress the aggregation of substrate proteins, they also form stable sHsp–substrate complexes. In the past, several regions and residues have been postulated to be involved in the chaperone function and complex formation of sHsps. The C-terminal region of αA-crystallin, Hsp25 and Hsp16.6, and Hsp69 of αA-crystallin and the N-terminal

![Figure 7. Complex formation of Hsp26ΔN30 and substrate proteins. A, Complexes of Hsp26ΔN30 and CS (continuous line) were analyzed after incubation of the proteins at a molar ratio of 1 : 1 (monomers) at 43 °C for 30 minutes. After incubation of the complex at 10 °C for ten minutes, it was applied to SEC. B, Complexes of Hsp26ΔN30 and Rho (continuous line) were analyzed after incubation of the proteins at a molar ratio of 1 : 1 (monomers) at 44 °C for 30 minutes. After incubation of the complex at 10 °C for ten minutes, it was applied to SEC. C, Stability of Hsp26ΔN30–Rho complexes (●) and Hsp26–Rho complexes (□) was determined using a Tosohaas TSK 4000 PW column as described in Materials and Methods. Complexes were formed by incubation of 3 μM Hsp26ΔN30 or Hsp26 and 4 μM rhodanese (monomers) at 44 °C for 30 minutes and subsequent incubation at 10 °C for ten minutes for complex stabilization. Subsequently, urea was added to the end concentrations indicated and samples were further incubated for 20 hours at 25 °C and finally separated on a pre-equilibrated column at 25 °C. For comparison, the dissociation of the Hsp26ΔN30 oligomeric peak alone was monitored (○). Incubation was performed as described without addition of Rho. Peak volumes were calculated and normalized, setting the signal of the complex without urea to 100.]
phenylalanine-rich region of α-crystallin have been suggested to be involved in chaperone activity. All of the proposed sites are of hydrophobic character and, in addition to chaperone function, also influence oligomerization of the respective sHsp. Thus, it is still unclear which of the proposed sites are necessary for chaperone function and which are involved in oligomer interactions, or if there is a substantial crosslink between those two functions. Comparisons between different sHsps are further limited by the low sequence conservation of the respective motifs, making variations in the substrate binding sites of different sHsps very likely.

The truncation of the N-terminal 30 residues of Hsp26 involves two of the potential motifs. First, the phenylalanine-rich region in Hsp26 is located to the very N terminus and is deleted in the truncated version. Second, a sequence stretch (GGLRGYAPRRQ) which might be the sequence corresponding to one of the two potential substrate binding motifs RTLGPFYPSR, described by Sharma et al., is enclosed in the truncated region. Nevertheless, Hsp26ΔN30 is still active as a chaperone, thus both sequence stretches are not essential for substrate recognition in Hsp26, but they might be involved in the formation of stable sHsp–substrate complexes.

In the case of M. jannaschii Hsp16.5, the N-terminal truncation of 31 residues resulted in a mutant defective in oligomerization and chaperone function. But, according to sequence homology the N-terminal region of Hsp16.5 would be comparable to the proposed middle domain of Hsp26. The deletion of the entire N-terminal region (including the middle domain) of Hsp26 resulted in a dimer without chaperone activity. This observation is in agreement with results on the isolated α-crystallin domain of Caenorhabditis elegans 16.2. M. jannaschii 16.5 and mouse Hsp25 all of which exhibited no chaperone activity. Interestingly, the truncation of only the 30 N-terminal residues of Hsp26 still allows suppressing the aggregation of substrate proteins like CS and Rho. However, Hsp26ΔN30–substrate complexes are less stable than Hsp26–substrate complexes. This observation might also explain the slight increase in the ability of Hsp26ΔN30 to decelerate the inactivation of CS indicating a more transient interaction of Hsp26ΔN30 with its substrate proteins compared to Hsp26.

Third, based on these observations and on studies on a Hsp26 mutant lacking the whole N-terminal region, we propose that both parts of the N-terminal region might cooperate in the chaperone function of Hsp26. The second part of the N-terminal region seems to be essential for the suppression of the aggregation of substrate proteins, while the N-terminal domain is important for the formation of stable Hsp26–substrate complexes.

**Materials and Methods**

**Materials**

Mitochondrial citrate synthase (CS) from pig heart (EC 4.1.3.7) was purified as described. Acetyl-CoA, bovine serum albumin (BSA), ferritin, catalase, aldolase and chymotrypsin were obtained from Roche Biochemicals (Mannheim, Germany). Bovine insulin and rhodanese (Rho) were from Sigma (St Louis, USA). The concentrations for all proteins given in the text refer to monomers, if not indicated otherwise. The Hsp26–expressing yeast strain was a kind gift from Dr S. Lindquist (Whitehead Institute, Boston, USA). Hsp26 was expressed and purified according to Haslbeck et al.

**Expression and purification of Hsp26ΔN30**

HSP26ΔN30 was amplified from genomic yeast DNA via PCR and cloned into pQE60 (QIAGEN, Hilden, Germany) fusing a C-terminal poly-histidine tag. Hsp26ΔN30 was expressed in the *Escherichia coli* strain M15 (QIAGEN, Hilden, Germany). Cells were grown in Lenox media (Roth, Karlsruhe, Germany) until *A*~600~ = 0.8, induced with 2 mM IPTG and shifted to 25 °C for 16 hours. Cells were harvested, washed once with buffer A (40 mM Hepes–KOH, 50 mM KCl (pH 7.4)) and lysed with a BasicZ cell disrupter (Constant Systems, Warwick, UK). The cell lysate was centrifuged (46,000 g, 45 minutes at 4 °C) and the soluble extract was applied to a 10 ml Ni-NTA column (QIAGEN, Hilden, Germany) operated as described in the suppliers manual under non-denaturing conditions. Eluted fractions were pooled and dialyzed against buffer A with 5 mM EDTA and loaded onto a Resource Q ion exchange column (Amersham Biosciences, Freiburg, Germany). The protein was eluted with a KCl gradient from 50 mM to 500 mM. The KCl eluate was further purified on a 16/60 Sephadex 200 pg gel filtration column (Amersham Biosciences, Freiburg, Germany), equilibrated in buffer A with 200 mM KCl. Hsp26ΔN30-containing fractions were pooled, dialyzed against buffer A with 50 mM KCl and concentrated by ultrafiltration.

**Partial proteolytic digestion and mass spectrometry**

For partial protein digestion, 20 μg of Hsp26 were digested with 5 ng of proteinase K for five minutes at 25 °C. The digestion products were analyzed by SDS-PAGE or subsequently purified using C4 Zip Tips (Qiagen, Hilden, Germany) and analyzed in a Biflex-II mass spectrometer (Bruker Daltonik, Bremen, Germany) operated in linear mode. To analyze the observed bands on SDS-PAGE, spots were excised and digested following the protocol of Schäfer et al. Sample preparation for MALDI-MS was performed, using C18 ZipTips (Qiagen, Hilden, Germany) following the manufacturer’s protocol.
Data analysis was performed using Mascot (Matrix Science, London, UK).

Circular dichroism spectroscopy

Far-UV CD spectra were recorded using a Jasco J-715 spectropolarimeter (Jasco, Groß-Umstadt, Germany) with a PTC 343 Peltier unit. The experiments were carried out in quartz cuvettes with 0.1 cm path length. Far-UV spectra were recorded from 190 nm to 260 nm in 50 mM potassium phosphate (pH 7.4) at 20 °C; 16 spectra were accumulated and all spectra were buffer-corrected. Thermal unfolding was recorded at 218 nm. The applied heating rate was 30 °C per hour. Structural elements were calculated using the CD spectra deconvolution CDNN software.

To monitor differences in the chemical stability of Hsp26ΔN30 and Hsp26ΔN30–rhodanese complexes, urea unfolding transitions were performed. A 0.25 mg/ml of protein were incubated for 20 hours at 25 °C in 40 mM Hepes–KOH (pH 7.4) containing different concentrations of urea. The unfolding transitions were analyzed using the Scientist software Micromath.

CS assays

Light scattering was used to examine the influence of Hsp26ΔN30 on the thermal aggregation of CS; 15 μM CS was diluted 1 : 5 in 40 mM Hepes–KOH (pH 7.5), equilibrated at 43 °C in the presence and in the absence of Hsp26ΔN30. Aggregation kinetics were measured in a Jasco V-550 spectrophotometer (Jasco, Groß-Umstadt, Germany) in a stirred and thermostated quartz cell at 400 nm.

Inactivation of CS was performed as described. CS was incubated in the absence or presence of Hsp26ΔN30 in 40 mM Hepes–KOH (pH 7.5) at 43 °C. To determine CS activity, aliquots were taken at time points indicated and the activity was measured according to Šrere, in 50 mM Tris–HCl, 2 mM EDTA (pH 8.0) at 25 °C.

Thermal aggregation of rhodanese

Monomeric bovine rhodanese (30 kDa) was diluted to a final concentration of 1 μM in 40 mM sodium phosphate (pH 7.7) and incubated at 44 °C. Assays in the presence of 4 μM BSA served as a control for unspecific protein effects. Aggregation was monitored at 400 nm in micro cuvettes (160 μl) with a path length of 10 mm.

Electron microscopy and image processing

Samples of full-length and Δ30 Hsp26 were negatively stained with ammonium molybdate and imaged on a Tecnai T12 microscope at 120 kV and 42,000× under low dose conditions, with a defocus between 0.4 and 0.8 μm. Data were recorded on photographic film, which was digitized at 3.3 A/pixel. Around 2000 particles were collected for each sample. Using IMAGIC-V Software, these were aligned to the same center and then classified by multi-variante statistical analysis, which provided references for further rounds of alignment and classification, until stable class averages were obtained.

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Analytical size exclusion chromatography

Size exclusion HPLC (SEC) was performed using a Tosohaas TSK 4000 PW column (Tosohaas, Stuttgart, Germany) with a separation range of 10 kDa to 1500 kDa. The column was equilibrated in buffer A with 200 mM KCl and the flow rate was 0.5 ml/min. Hsp26ΔN30 was detected by fluorescence at an excitation wavelength of 275 nm and an emission wavelength of 326 nm using a FP 920 fluorescence detector (Jasco, Groß-Umstadt, Germany). Standard proteins from the molecular weight marker kit (Sigma, St Louis, USA) were used for calibration.

To analyze the stability of Hsp26 and Hsp26ΔN30–substrate complexes, SEC was performed at 25 °C or 43 °C in buffer A with 200 mM KCl and urea concentrations as indicated in the Figure legends. The sample volume was 100 μl. Complexes were formed by incubation of 3 μM Hsp26ΔN30 or Hsp26 and 4 μM rhodanese (monomers) at 44 °C for 30 minutes. After incubation the complexes were stabilized at 10 °C for ten minutes. Spectra were analyzed using the Sigma Plot software package (SPSS, Chicago, USA).

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Analysis of the N-terminal Domains of Hsp26


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