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Caenorhabditis elegans small heat-shock proteins Hsp12.2 and Hsp12.3 form tetramers and have no chaperone-like activity

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Abstract Four 12.2–12.6 kDa small heat-shock proteins (sHSPs) of *Caenorhabditis elegans* are the smallest known members of the sHSP family. They essentially comprise the characteristic C-terminal 'α-crystallin domain' of the sHSPs, having a very short N-terminal region, and lacking a C-terminal tail. Recombinant Hsp12.2 and 12.3 are characterized here. Far-UV CD spectra reveal, as for other sHSPs, predominantly a β-sheet structure. By gel permeation and crosslinking, they are the first sHSPs shown to occur as tetramers, rather than forming the usual large multimeric complexes. Exceptionally, too, both appear devoid of in vitro chaperone-like abilities. This supports the notion that tetramers are the building blocks of sHSP complexes, and that higher multimer formation, mediated through the N-terminal domains, is a prerequisite for chaperone-like activity.

Key words: Small heat-shock protein; Molecular chaperone; *Caenorhabditis elegans*

1. Introduction

Small heat-shock proteins (sHSPs) are present in all organisms studied, and form a diverse family of proteins with subunit molecular masses ranging from 12 to 43 kDa (for recent reviews, see [1–3]). Many of them are constitutively expressed, often developmentally regulated and reaching very high levels, while others are induced under various stress conditions. Overexpression of sHSPs in cultured cells confers thermotolerance [4]. The mechanism of this protective effect is unknown, but is likely to depend on the chaperone-like activity of the sHSPs [5], that is their ATP-independent capacity to prevent the aggregation of denaturing proteins. By stably binding misfolded proteins in the cell, the sHSPs may create a reservoir of non-native proteins that can be refolded to the native state, probably in cooperation with other, ATP-dependent molecular chaperones [6–8].

Structurally, the sHSP subunits are thought to consist of an N-terminal domain, which is variable in sequence and length, and an evolutionarily conserved C-terminal or 'α-crystallin' domain which terminates in a short and flexible C-terminal tail [3,9,10]. Subunits generally assemble into large heterogeneous complexes (150–800 kDa), possibly in a flexible micelle-like arrangement, with the hydrophobic N-terminal domains directed inward, and the polar C-terminal domains and tails extending into solution [11–14]. Preliminary X-ray crystallographic data of the 16.5 kDa sHSP of the archaebacterium *Methanococcus jannaschii* is now available [15]. It reveals a 24 subunit octahedral complex, formed by rotation around a threefold crystallographic axis of eight subunits in an asymmetric unit. Recently, too, cryo-electron microscopic studies have shown that αB-crystallin, a major vertebrate sHSP, forms roughly spherical 8–18 nm asymmetric complexes containing a large 3–10 nm cavity [16].

It is reasonable to assume that clues for the common structural and functional features of the sHSP family can be found in their conserved 80–100 residue α-crystallin domain, whereas the variable N-terminal domain and C-terminal tail modulate the specific properties of each individual type of sHSP. In that respect the four 12 kDa sHSPs of *Caenorhabditis elegans* are of special interest. These are the smallest known representatives of the family, essentially reduced to the core α-crystallin domain [3,17,18]. Their N-terminal 'domains' comprise a mere 25–26 residues, and C-terminal tails are altogether lacking. Surprisingly, one of these sHSPs from *C. elegans*, Hsp12.6, was recently reported to be monomeric and devoid of chaperone activity [18]. To further explore these prototypic sHSPs, we now characterized *C. elegans* Hsp12.2 and Hsp12.3, which are the most divergent of the four, displaying only 43% sequence identity.

2. Materials and methods

2.1. Cloning, expression and purification of Hsp12.2 and Hsp12.3

cDNA of Hsp12.2, designated as C14B9.1 in the *C. elegans* chromosome III sequencing project [18] (accession number L15181), was amplified from a mixed-stage cDNA library [19], using the 5′ primer 5′-GGAATTC**CATATG**TCCGCTATCGAGGTGAC-3′ (*Eco*RI and *Nde*I sites underlined and bold, respectively) and the 3′ primer 5′-ATCTGGGATCCTTAAGCCTTCTTGGAAGCAG-3′ (*Bam*HI site underlined). The cDNA was subcloned into the *Eco*RI-*Bam*HI digested pGEM7Zf(+) vector, sequenced and subsequently cloned into the *Nde*I-*Bam*HI digested pET3a expression vector. Hsp12.3 (accession number Z68342 [18]) was amplified using the 5′ primer 5′-AGT-**CATATG**TCTGTTGCTATTGATCAC-3′ (*Nde*I site in bold) and the 3′ primer 5′-GCTGGATCCTTACTTTTTCTTGTTTCCGGAGA-TGTG-3′ (*Bam*HI site underlined), and cloned into a modified pRSET A vector lacking the polyhistidine tag, as described earlier [18]. The expression vectors containing Hsp12.2 and Hsp12.3 cDNA were transformed into *Escherichia coli* strains BL21(DE3)pLysS and BL21(DE3), respectively. Induction, cell lysis, and fractionation were essentially performed as described by Merck et al. [20]. Recombinant

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Abbreviations: sHSP, small heat-shock protein; rec-, recombinant; SP buffer, sodium phosphate buffer; DTT, dithiothreitol; CD, circular dichroism; BSA, bovine serum albumin

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Hsp12.2 (rec-Hsp12.2) was largely present in the soluble fraction, and purified over a Fast Flow DEAE-Sepharose anion exchange column (Pharmacia-LKB), eluted with TEN_{50} buffer, pH 7.7. Rec-Hsp12.2 was present in the flow-through. Fractions containing rec-Hsp12.2 were pooled, concentrated and stored at 4°C in the presence of 0.05% NaN_3. Purification of recombinant Hsp12.3 (rec-Hsp12.3) was essentially as described for rec-Hsp12.6 by Leroux et al. [18]. Since rec-Hsp12.2 and rec-Hsp12.3 were not sequenced, it remains uncertain whether the N-terminal methionine is still present.

2.2. Circular dichroism spectroscopy and heat stability measurement

Circular dichroism spectra were obtained on a Jasco J-715 spectropolarimeter. Spectra shown are the average of four scans with a scan rate of 20 nm/min and a quartz cell length of 1 mm. Experiments were performed using a protein concentration of 250 μg/ml in 0.1 M Na_2SO_4, 20 mM NaP_i, pH 6.9 (SP buffer) at 20°C. Native rec-Hsp12.2 and rec-Hsp12.3 were diluted with SP buffer to a final concentration of 0.5 mg/ml. Temperature was raised (1°C/min) and absorption at 360 nm was measured every 30 s, using a Perkin-Elmer Lambda 2 UV/Vis spectrophotometer connected to a thermostatted circulation waterbath and a thermocouple to register the sample temperature.

2.3. Size exclusion chromatography and crosslinking of rec-Hsp12.2 and rec-Hsp12.3

The complex size of native and crosslinked rec-Hsp12.2 was determined using an LKB Bromma HPLC system in conjunction with a Superose 12 HR 10/30 prepacked size exclusion column (30×1 cm, Pharmacia-LKB). Protein samples were 0.2 mg in 1 ml of SP buffer. Elution was performed in the same buffer, at a flow rate of 0.5 ml/min, and absorbance was measured at 278 nm. Fractions were analyzed by SDS-PAGE [21]. The size of rec-Hsp12.3 was estimated by chromatography over a 100×1.5 cm Sephacryl S-200 HR column, as described in [18]. Crosslinking of rec-Hsp12.2 was performed as described by Siezen et al. [22] with minor modifications. The homobifunctional crosslinker dimethylsuberimidate (5 mM) was used, with 100 μg/ml rec-Hsp12.2 and 10 mM dithiothreitol (DTT) in 0.2 M triethanolamine, pH 8.0. Total volume was 2 ml. The reaction was stopped after 2 h at room temperature by adding glycine up to a final concentration of 50 mM. Crosslinking of 1 μM rec-Hsp12.3 with 2 mM bis(sulfosuccinimidyl)suberate was carried out in the presence or absence of 16 μM bovine serum albumin (BSA) and the reaction products detected by Western blot analysis with an anti-Hsp12.6 polyclonal antiserum [18].

2.4. Chaperone assays

Two methods were used to assess the capacity of rec-Hsp12.2 and rec-Hsp12.3 to protect unfolding proteins from aggregating. In the insulin-protection assay [23], different amounts rec-Hsp12.2 and rec-Hsp12.3 were incubated with 250 μg bovine pancreas insulin (Sigma) at 40°C for 5 min, in a total volume of 0.98 ml SP buffer. Denaturation of insulin was then induced by adding 20 μl 1 M DTT, and turbidity was measured at 360 nm for 15 min at 40°C, as described above. In the citrate synthase assay [6], various amounts of rec-Hsp12.2 and rec-Hsp12.3 were incubated for 5 min at 43°C in a volume of 2 ml 40 mM HEPES-KOH, pH 7.5. Citrate synthase (Boehringer) was then added to a final concentration of 75 nM, and scattering was measured on a Hitachi F-3000 spectrofluorimeter (excitation and emission wavelengths 500 nm; band width 3 nm; equipped with a circulating thermostatted waterbath) at 43°C for 15 min.

3. Results and discussion

3.1. Isolation, secondary structure, and stability of rec-Hsp12.2 and rec-Hsp12.3

To obtain pure recombinant rec-Hsp12.2 and rec-Hsp12.3 proteins, the respective cDNAs were amplified with specific primers from mixed-stage *C. elegans* cDNA libraries, and cloned into vectors for expression in *E. coli*. Both proteins were found in the soluble fraction of the bacterial lysate, although rec-Hsp12.2 was also partially present in the insoluble pellet. The proteins were isolated from the soluble fraction, and purified to near-homogeneity (>95%). Proper folding of the recombinant proteins was confirmed by far-UV circular dichroism spectroscopy (Fig. 1A). The spectra for rec-Hsp12.2 and rec-Hsp12.3 both have negative ellipticity, with minima at approximately 215 nm and 218 nm, respectively, which is typical for β-sheet structure. These results are in agreement with secondary structure predictions made for Hsp12.2 and Hsp12.3 [18], and are comparable to the far-UV CD spectra observed for other sHSPs [1,2].

The partial presence of rec-Hsp12.2 in the insoluble fraction after expression in *E. coli* might indicate a lower stability as compared to rec-Hsp12.3. We therefore determined the thermostability of rec-Hsp12.2 and rec-Hsp12.3 by incubating them at increasing temperatures in a thermostatted UV/VIS-spectrophotometer, and measuring the turbidity at 360 nm (Fig. 1B). While rec-Hsp12.3 remained stable at temperatures as high as 65°C for more than 30 min, rec-Hsp12.2 came fully out of solution around 55°C. A far-UV CD spectrum of heat-treated rec-Hsp12.3 (Fig. 1A) showed that only minor irreversible changes in secondary structure had occurred after heating for 30 min at 65°C.

3.2. Rec-Hsp12.2 and rec-Hsp12.3 form tetramers

Size exclusion chromatography showed that rec-Hsp12.3 eluted as a rather broad peak, indicative of some size heterogeneity, centered around an estimated mass of 39 kDa (Fig. 2A). A similar result was obtained for rec-Hsp12.2 (see Fig. 2C). These masses would suggest a trimeric structure, which is unexpected because all other sHSPs, with the exception of the related Hsp12.6 [18], form much larger complexes. To establish the actual number of subunits in the oligomers of rec-

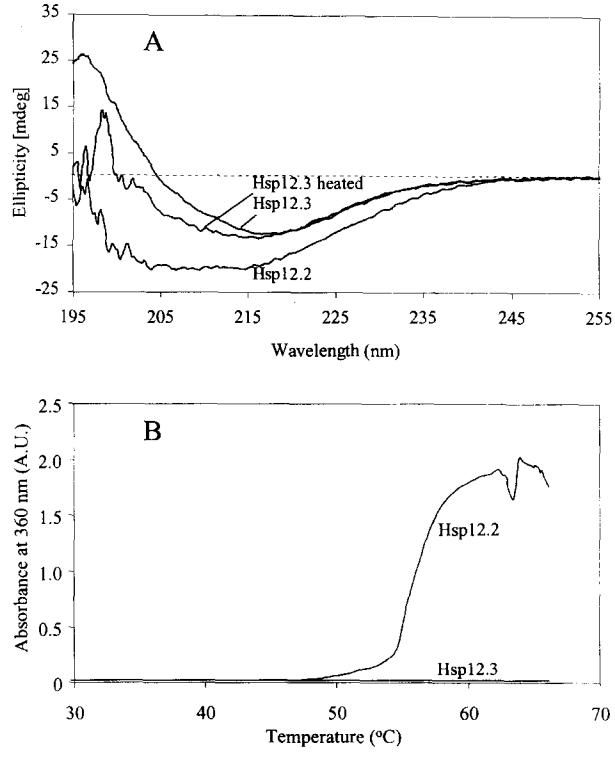

Fig. 1. A: Far-UV circular dichroism spectra of rec-Hsp12.2, rec-Hsp12.3, and rec-Hsp12.3 after heating for 30 min at 65°C. B: Thermostability of rec-Hsp12.2 and rec-Hsp12.3, monitored by measuring the turbidity at 360 nm.

Hsp12.2 and rec-Hsp12.3, we treated them with the homobifunctional crosslinking agents dimethylsuberimidate and bis(sulfosuccinimidyl)suberate, respectively. Crosslinking of rec-Hsp12.3 yielded mainly dimers, some trimers and a heterogeneous group of tetramer-like bands (Fig. 2B, lane 1). In the presence of 10-fold excess BSA, to avoid non-specific crosslinking, the trimers decreased and the tetramer became more homogeneous (Fig. 2B, lane 2). In the case of rec-Hsp12.2, crosslinked dimers, trimers and tetramers were all obtained at higher levels (see Fig. 2C).

The trimer-like elution of rec-Hsp12.2 and 12.3 during size exclusion chromatography might reflect an equilibrium between tetramers, trimers, dimers and even monomers. In that case, one would expect that the covalently crosslinked tetramers elute earlier than the trimers or dimers. Crosslinked rec-Hsp12.2 was therefore applied onto a Superose 12 column. Again, a single homogeneous peak was obtained, eluting at a position corresponding to the estimated mass of a trimer (36 kDa) (Fig. 2C). SDS-PAGE showed that all three crosslinking products were present over the width of the peak, with tetramers somewhat more abundant in the earlier fractions, and monomers in the last eluting fractions (Fig. 2C). Since the actual mass of covalently crosslinked tetrameric rec-Hsp12.2 necessarily is 48.9 kDa, it appears that the oligomer size of rec-Hsp12.2 cannot accurately be estimated by size exclusion chromatography. We conclude that rec-Hsp12.2 and rec-Hsp12.3 largely occur as tetramers, probably in slow equilibrium with trimers, dimers and/or monomers.

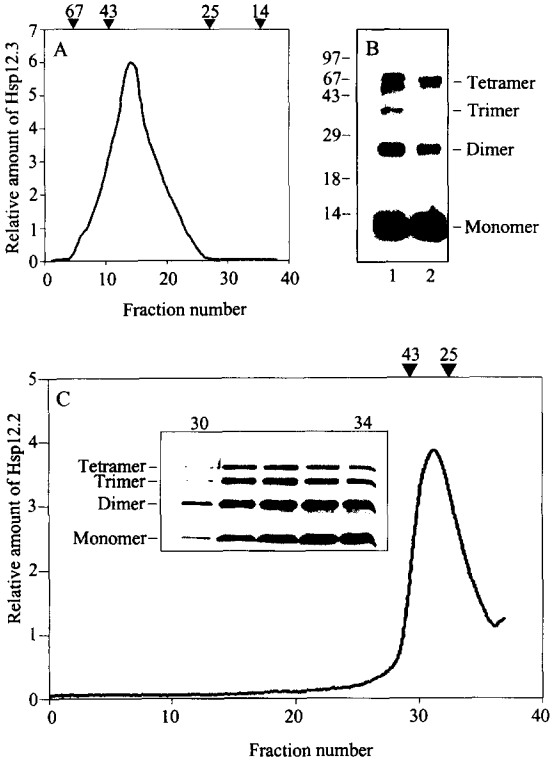

Fig. 2. Determination of the oligomeric structure of rec-Hsp12.2 and rec-Hsp12.3. A: Elution profile of purified rec-Hsp12.3 on a Sephacryl S-200 HR column. Arrowheads indicate the elution positions of molecular mass markers. B: Western blot of rec-Hsp12.3 after crosslinking by bis(sulfosuccinimidyl)suberate, in the absence (lane 1) or presence (lane 2) of BSA. Positions of molecular mass markers are indicated. C: Elution profile of rec-Hsp12.2 on a Superose 12 column after crosslinking by dimethylsuberimidate. The insert shows the SDS-PAGE pattern of fractions 30–34, stained with Coomassie brilliant blue.

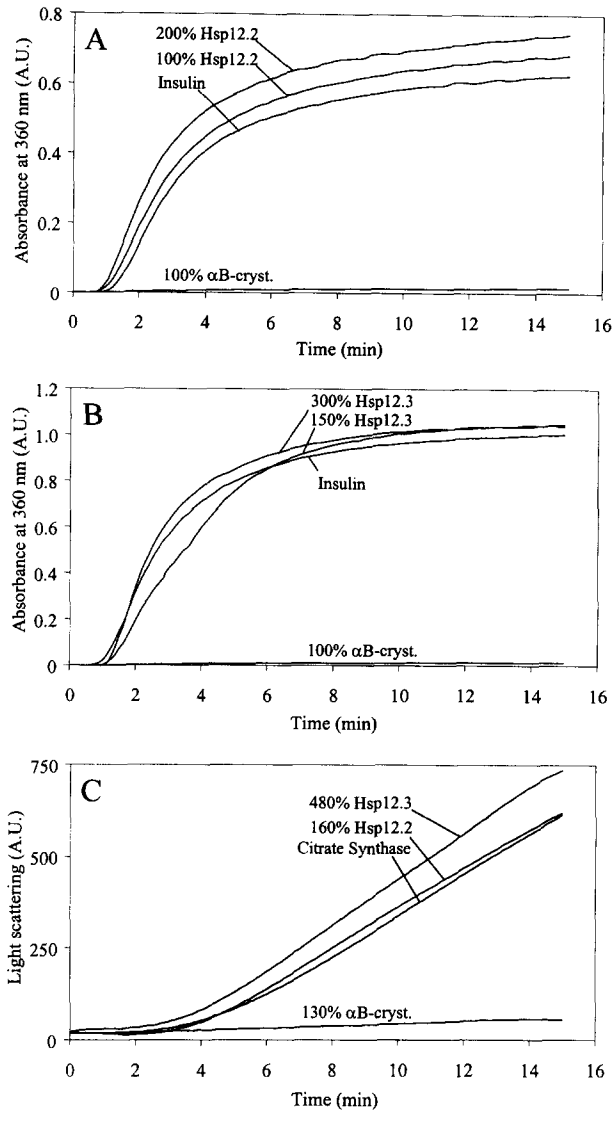

Fig. 3. Chaperone assays of rec-Hsp12.2 and rec-Hsp12.3. A, B: Reduction-induced aggregation of insulin B-chain at 40°C as a function of time in the presence and absence of various amounts of rec-Hsp12.2 (A) and rec-Hsp12.3 (B) and αB-crystallin. sHSP to insulin mass ratios are indicated. C: Temperature-induced aggregation of citrate synthase at 43°C in the absence and presence of rec-Hsp12.2, rec-Hsp12.3 and αB-crystallin.

3.3. *Rec-Hsp12.2 and rec-Hsp12.3 lack chaperone-like activity*

In view of the relatively lower heat stability of rec-Hsp12.2 as compared with rec-Hsp12.3, we could not perform the original heat-protection chaperone assay at 58°C [5]. However, the reduction-induced aggregation of insulin, performed at 40°C [23], appeared not to be prevented by the presence of up to 100–300% mass ratios of rec-Hsp12.2 or rec-Hsp12.3 to insulin (Fig. 3A,B, respectively). In contrast, at a 100% mass ratio, αB-crystallin provided complete protection. To confirm the apparent lack of chaperone-like capacity of rec-Hsp12.2 and rec-Hsp12.3, we also tested their ability to prevent the aggregation of thermally unfolding citrate synthase, performed at 43°C [6]. Again, the presence of an excess of rec-

Hsp12.2 or rec-Hsp12.3 did not prevent the aggregation of citrate synthase, whereas αB-crystallin was very effective (Fig. 3C).

3.4. Lessons from rec-Hsp12.2 and rec-Hsp12.3

Rec-Hsp12.2 and rec-Hsp12.3 are the first sHSPs reported to occur as tetramers, while not forming larger complexes. Tetrameric and dimeric arrangements have earlier been implicated as building blocks for larger sHSP complexes. Calorimetric studies proposed a dimer structure as the minimum cooperative subunit of mammalian Hsp25 [24]. The isolated C-terminal domain-with-tail of αA-crystallin assembles into tetramers, and also resembles rec-Hsp12.2 and 12.3 in that it lacks chaperone activity [20]. Moreover, in the presence of 1% deoxycholate, αA-crystallin complexes dissociate into 80 kDa tetramers, without altering the secondary and tertiary structure [25]. Based on tetrameric building blocks, a rhombododecahedral model, with two-, three- and fourfold symmetries, has been proposed for α-crystallin [26]. In light of the most recent X-ray [15] and cryo-EM [16] data, we can envisage that tetrameric building blocks, as exemplified by the 12 kDa sHSPs, form the wall of a cage. The more hydrophobic N-terminal domains are likely to provide the necessary interactive surfaces to assemble the higher order complexes.

It is tempting to attribute the inability of rec-Hsp12.2 and rec-Hsp12.3, as well as the αA-crystallin C-terminal domain-with-tail, to form larger complexes than tetramers, to the near or complete absence of an N-terminal domain. Also for *C. elegans* rec-Hsp16.2, it has been observed that shortening of the N-terminal domain by 15 residues – which gives its N-terminal domain the same length as that of the Hsp12 family – abrogates its ability to form large multimeric complexes [13]. It thus appears that an N-terminal domain of sufficient length is generally required for sHSP multimerization. It is of interest that *C. elegans* Hsp12.6, which is 48 and 67% identical to Hsp12.2 and 12.3, respectively, did not seem to form oligomers, but rather was considered to be monomeric based on sedimentation velocity data [18]. It may well be that the equilibrium between mono- and oligomers is not positioned equally for all rec-Hsp12 members, and that only a very small fraction of rec-Hsp12.6 forms di- and tetramers (see Fig. 5, lane 1 in [18]). It is also possible that the crosslinking efficiency of different family members could be variable, resulting in a lesser degree of crosslinking for rec-Hsp12.6, especially at lower concentrations of the protein.

The properties of rec-Hsp12.2, rec-Hsp12.3 and the C-terminal domain-with-tail of αA-crystallin, as well as the truncation mutants of *C. elegans* Hsp16-2 all indicate, too, that higher complex formation, beyond the tetramer, is also required for chaperone-like activity. This may agree with the observation that only the large complexes of Hsp25/Hsp27 are able to confer in vivo protection against oxidative stress [27,28]. Although the sites of interaction of unfolded substrates with sHSPs are likely to be within or very close to the conserved α-crystallin domain [7,29], the N-terminal domain has also been implicated in substrate binding [14]. In addition to accessible hydrophobic binding sites, it appears that the presence of flexible polar C-terminal extensions improves the chaperone capacity of sHSPs by enhancing the solubility of the sHSP-substrate complex [13,14]. The loss in the *C. elegans* Hsp12 family of a sufficiently large N-terminal domain, and the absence of a C-terminal tail, would then explain both the inability to form larger multimers and the lack of in vitro chaperone-like activity.

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