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Recombinant expression and in vitro refolding of the yeast small heat shock protein Hsp42☆

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Abstract

Small Hsps represent a variation on the theme of protection of proteins from irreversible aggregation by reversible interaction with chaperone proteins. While different sHsps are highly heterogeneous in sequence and size, the common trait is the presence of a conserved α-crystallin domain. In addition sHsps assemble into large oligomeric complexes where dimers represent the basic building blocks. Hsp42, a member of the sHsp family in the cytosol of *S. cerevisiae*, forms ordered oligomers with a barrel-like structure. Here, we present the recombinant expression and purification of Hsp42. We demonstrate, that Hsp42 is expressed in inclusion bodies and can be resolubilized and folded to correct, active oligomers. This indicates that in contrast to thermal unfolding, the chemical disassembly and unfolding of Hsp42 is fully reversible. In comparison to the purification of mature Hsp42 from yeast, its recombinant expression leads to a substantial increase in the yield of the protein and to a reduction of contamination caused by aggregation prone proteins complexed by Hsp42. In addition, the recombinant Hsp42 is fully active as a chaperone in an energy independent manner.

Keywords: Hsp42; sHsp; Protein folding; Protein purification; Chaperone activity

1. Introduction

sHsps show a characteristic heterogeneity in sequence and size. The common trait is the conserved C-terminal domain, the so-called α-crystallin domain. The term refers to the most prominent family member, the eye-lens protein α-crystallin [1]. However, all sHsps investigated up to now form large oligomeric complexes, mainly of 12–42 subunits [2–6]. Structural analysis of several members of the family revealed hollow particles with openings, where the basic building block of the oligomer is a dimer [7–11]. Several species specific variations of this common scheme have been described recently. For example, wheat Hsp16.9 assembles into a dodecameric double disk, each disk organized as a trimer of dimers [8]. 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 [11–16]. sHsps have been included in the class of molecular chaperones because they bind specifically to unfolded proteins in vitro and prevent their aggregation by forming large, stable sHsp–substrate complexes of globular shape [9,10,17–21].

Although the non-native substrate protein is not released spontaneously, these complexes are not dead-ends. In the presence of ATP, Hsp70 and/or Hsp104 chaperones are able to promote the folding of the bound substrate proteins to the native state [18,19,22–25]. In vivo the emerging picture is that sHsps bind non-native proteins under stress conditions. Binding prevents the formation of aggregates and enables the subsequent refolding by Hsp70, Hsp104 or other ATP-dependent chaperone systems [22–27].

The cytosol of *S. cerevisiae* contains at least two chaperones of the sHsp family with partially overlapping function. Hsp42 acts as a chaperone both at physiological and heat shock

Abbreviations: Rho, Rhodanese; BSA, bovine serum albumin; DTT, dithiothreitol; (s)Hsp, (small) heat shock protein; SEC, size exclusion chromatography; PAGE, polyacrylamide gel electrophoresis; OD, optical density; EDTA, ethylenedinitrilotetraacetic acid

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temperatures [10], while Hsp26 is significantly activated as a chaperone at elevated temperatures [9]. Together they prevent aggregation of cytosolic proteins under physiological and stress conditions.

Like α-crystallin and other sHsps, Hsp42 is a dynamic oligomer and single particle analysis of negative-stain preparations revealed a predominant population of ring-like complexes [10]. The averaged projection image of Hsp42 strongly resembles the structural organization of Hsp16.9 from wheat [8], a double disc of two hexameric rings.

Hsp42 is a promiscuous chaperone. It is able to suppress the aggregation of a broad variety of substrate proteins in vitro and is able to bind at least 30% of the yeast cytosolic proteins [10]. This promiscuousness is one of the major problems in purifying mature Hsp42 from the yeast cytosol. Due to its high substrate binding activity various contaminants are stably bound and hard to separate.

Here we present a strategy to express Hsp42 in *E. coli* and purify it under denaturing conditions. Surprisingly, Hsp42 could be refolded efficiently in vitro, resulting in correctly assembled and functional oligomers. Furthermore, testing the functionality of Hsp42 we were able to show for the first time that Hsp42 does not hydrolyze ATP and, like other sHsps, acts in an energy independent manner.

2. Experimental

2.1. Materials

Rhodanese was obtained from Sigma (St. Louis, USA). The Hsp26 and Hsp104 expression plasmids were a kind gift of Dr. S. Lindquist (Whitehead Institute, Boston, USA). Hsp26 and Hsp104 were expressed and purified according to Haslbeck et al. [9] or Schirmer and Lindquist [28], respectively. For comparison, native Hsp42 was purified from yeast as described elsewhere [10]. For ATPase activity measurements, mature Hsp42 was further purified in analytical scale on a 0.2 ml ATP agarose column, where Hsp42 was found in the flow through, followed by an additional size exclusion chromatography step (buffer, 100 mM Tris/HCl, 150 mM NaCl, 1 mM DTT, pH 8.0; TSK 4000 PW; TosoHaas, Stuttgart, Germany). Densitometric analysis of Coomassie stained SDS-PAGE gels was performed using the ImageMaster 1D software (Amersham Biosciences, Freiburg, Germany).

2.2. Cloning and expression of recombinant Hsp42

HSP42 was amplified from genomic yeast DNA via PCR with the primers GACTGGATCCAGTTTTTATCAACAATCCCTATCTC, CATGAAGCTTTCAATTTTCTACCGTAGGGTTGG and cloned into pQE60 (Qiagen, Hilden, Germany) via the introduced *Bam*HI and *Hin*dIII restriction sites. The protein was expressed in *E. coli* M15 (Qiagen, Hilden, Germany) cultivated at 37 °C. Induction of the expression plasmids was performed at $OD_{600} = 0.5–0.8$ with 2 mM IPTG shifting the cells to 30° subsequently after induction.

2.3. Isolation and solubilization of inclusion bodies

The cell pellet was resolved in 50 mM Tris/HCl, 1 mM EDTA, 5% glycerol, pH 7.4, and incubated with 1.5 mg of lysozyme/g of cells for 30 min at 4 °C. Cells were lysed with a disrupter (Constant Systems). The lysed cells were incubated with 10 μg of DNase I and 3 mM $MgCl_2$ for 30 min at 25 °C to digest the DNA. To isolate the inclusion bodies 0.5 volumes of 60 mM EDTA, 6% Triton X-100, 1.5 M NaCl, pH 7.0 were added and the solution was incubation for 30 min at 4 °C. The inclusion body pellet was centrifuged (40,000 × *g*, 30 min, 4 °C) and washed in 50 mM Tris/HCl, 20 mM EDTA, pH 7.4. The pellet was resolubilized in 50 mM Tris/HCl, 8 M urea, 100 mM DTT, pH 7.4, and incubated for 2 h at 25 °C.

2.4. Chromatography

All chromatography steps were performed on an Äkta FPLC system (Amersham Biosciences, Uppsala, Sweden). Ion exchange chromatography was performed using five column volumes (CV) for equilibration, loading of sample, 5–10 CV for washing and linear gradients of 15–25 CV. Buffer conditions, flow rates and column types are stated in results.

2.5. Electrophoresis

SDS-PAGE was performed in a SE 250 Mighty Small electrophoresis system according to the manufacturers' protocol (Amersham Biosciences, Freiburg, Germany) at a constant current of 30 mA per gel. Coomassie staining was performed as described elsewhere [29].

2.6. Immunoblotting

Proteins were separated by SDS-PAGE, and transferred to PVDF membranes. The immunodetection was performed using affinity-purified polyclonal rabbit antiserum raised against purified Hsp42. For detection, a horse radish peroxidase-linked secondary conjugate (Sigma, St. Louis, USA) was used and reactive bands were visualized by Enhanced Chemiluminescence (ECL) Detection Reagents (Amersham Biosciences, Freiburg, Germany). Alternatively, Cy5 conjugated antibodies against rabbit IgG (Amersham Biosciences, Freiburg, Germany) were used for detection and analysis of Hsp42 quantities [10]. For calculation of protein contents, dilution series of purified Hsp42 were used for calibration.

2.7. Circular dichroism spectroscopy

CD spectra were recorded using a Jasco J-715 spectropolarimeter (Jasco, Groβumstadt, Germany). The experiments were carried out in quartz cuvettes with 0.02 cm path length. Far UV-spectra were recorded from 200 to 250 nm in 50 mM potassium phosphate (pH 7.4) at 20 °C; 16 spectra were accumulated and all spectra were buffer-corrected. Structural elements were calculated using the CDNN software (http://www.bioinformatik.biochemtech.uni-halle.de/cdnn).

2.8. 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 10–1500 kDa without pre-column. Buffer conditions were 100 mM Tris/HCl, 150 mM KCl, 2 mM EDTA, 1 mM DTT, pH 7.4 and a flow rate of 0.5 ml/min was used. Hsp42 was detected by fluorescence at an excitation wavelength of 275 nm and an emission wavelength of 307 nm using a FP 920 fluorescence detector (Jasco, Groβumstadt, Germany). Standard proteins from the molecular weight marker kit (Thyroglobulin, 669 kDa; Apoferritin, 443 kDa; BSA, 66 kDa; Sigma, St. Louis, USA) were used for calibration.

2.9. Thermal aggregation of Rhodanese

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

2.10. ATPase activity

The hydrolysis of ATP was measured using a colorimetric, Malachite Green based assay as described by Schirmer and Lindquist [28]. Values were calibrated against KH_2PO_4 standards and corrected for phosphate release in the absence of protein. Hsp104 was used as activity control.

3. Results

First, we designed a recombinant expression system for Hsp42 in *E. coli*. Therefore, we cloned the Hsp42 encoding gene into pQE60 (Qiagen, Hilden, Germany). In this expression vector the Hsp42 gene is under the control of a T5 promotor regulated by an IPTG inducible operator sequence. For expression, *E. coli* M15 (pRep4) cells (Qiagen, Hilden, Germany) were transformed with the Hsp42 expression plasmid.

As described in Fig. 1, Hsp42 could be expressed in *E. coli*, but unfortunately the protein was insoluble (Fig. 1B and C). Variations of the expression temperature and the inductor concentrations did not improve the solubility of the protein (data not shown).

Therefore, in a further set of experiments we tested the possibility of purifying the insoluble protein and refolding it to its active and oligomeric state. After expression, the cells were lysed and inclusion bodies were isolated and solubilized as described in Section 2. To monitor the content and initial status of purity of Hsp42, the resolved inclusion bodies were analyzed by SDS-PAGE (Fig. 2). In comparison to total cell extracts a substantial purification was already achieved by the described inclusion body isolation and solubilization strategy.

The efficiency of in vitro folding of proteins from inclusion bodies is influenced by many parameters such as temperature, pH ionic strength or protein concentrations and has to be determined for each protein empirically [31–34]. In the case of Hsp42, the protein must first refold and then associate to the correct oligomeric state. We tested the refolding of the protein on an analytical scale by diluting it into a subset of different refolding buffers (Fig. 3). For each refolding experiment a total of 300 μg solubilized protein (concentration 6 mg/ml) was diluted to a final concentration of 30 μg/ml (Fig. 3). After incubation at 10 °C for 16 h the samples were centrifuged to remove precipitates and the amounts of soluble Hsp42 in the supernatant were analyzed by SDS-PAGE and immunoblot. Surprisingly, we were able to gain soluble Hsp42 under various buffer conditions (Fig. 3), but Tris buffers proved to be more suited than sodium phosphate buffers or HEPES buffers (Fig. 3; data not shown). In the absence of DTT no refolding was observed, indicating that the single cysteine in Hsp42 must be kept in the reduced state, and small amounts of EDTA were necessary to circum-

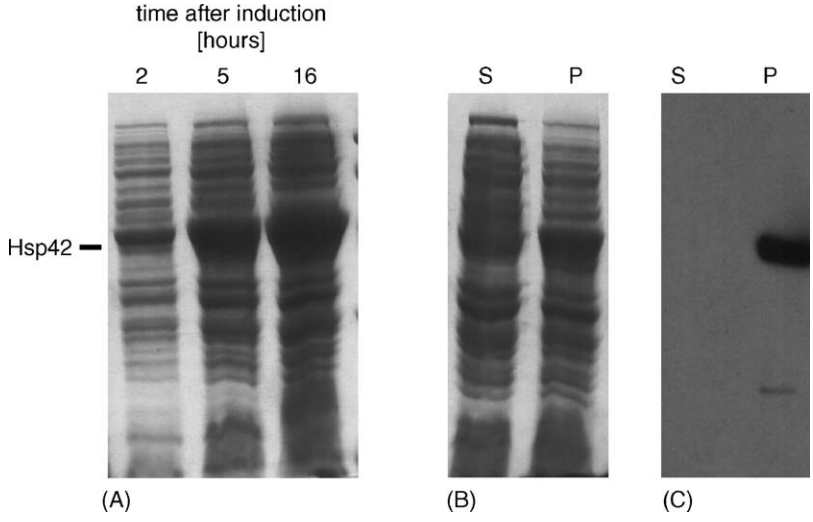

Fig. 1. Kinetic analysis of Hsp42 expression. (A) Coomassie stained SDS-PAGE of cell samples taken at indicated time points after induction. A total of 1×10^4 cells were lysed and loaded per lane. (B and C) A total of 1×10^9 cells were lysed 5 h after induction. Cells were lysed by sonification and the insoluble content (P) was pelleted by centrifugation. Soluble (S) and insoluble contents were analyzed by Coomassie stained SDS-PAGE (B) or immunoblot (C).

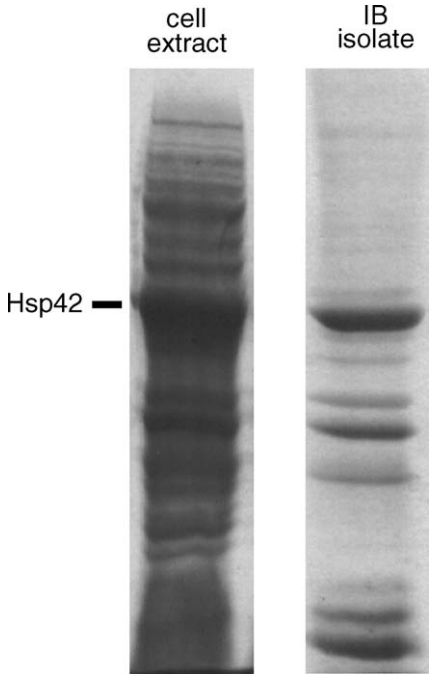

Fig. 2. Isolation of Hsp42 inclusion bodies. Coomassie stained SDS-PAGE comparing total cell extract after 16 h of Hsp42 expression at 30 °C and the isolated and solubilized inclusion body preparation.

vent degradation. Thus, all refolding buffers contained 1 mM DTT and 2 mM EDTA. According to our refolding screen, ionic strength and pH seemed to be the major parameters influencing the solubility of Hsp42 (Fig. 3). The presence of the amino acid arginine displayed stabilizing influences but was not essential for folding of Hsp42 and had no additive or synergistic effect on the refolding yield (Fig. 3D). The addition of low concentrations of detergents like Brij 58 (Fig. 3D), Triton X-100 or Tween 20 also had no additional positive effects. The addition of glycerol (2.5–10%) increased the refolding yield nearly twofold, but unfortunately several degradation bands of the Hsp42 became apparent under these conditions (data not shown). Having established a suitable refolding buffer allowing the folding of ∼15% of the Hsp42 solubilized from the inclusion bodies, we tried to further increase the refolding efficiency by increasing the concentration of protein in the folding buffer (100 mM Tris/HCl, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, pH 8.0) from 30 μg/ml by step wise addition of further 30 μg every 30 min, to a final concentration of 150 μg/ml. This procedure allowed us to increase the final concentration of refolded Hsp42, keeping the folding yield constant at ∼15% of the solubilized Hsp42 (data not shown).

For further purification we loaded the soluble, refolded fraction on a 6 ml Resource Q anion exchange column (Amersham Biosiences, Freiburg, Germany) equilibrated in 100 mM Tris/HCl, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, pH 8.0 at a constant flow rate of 1 ml/min at 4 °C. Bound proteins were eluted in a gradient of 150–750 mM NaCl (Fig. 4). Along this gradient, Hsp42 eluted as a wide peak from 170 to 350 mM NaCl (Fig. 4). In this chromatography step, no significant purification but a significant concentration of Hsp42 was achieved. The Hsp42 containing fractions were pooled and separated on a 16/60 Sephacryl 300 pg column (Amersham Biosciences, Freiburg, Germany) equilibrated in 100 mM Tris/HCl, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, pH 8.0 at a constant flow rate of 1 ml/min at 4 °C (Fig. 4). Due to its high molecular mass, oligomeric Hsp42 eluted very early, immediately after the void volume of the column. In addition to contaminating proteins, in this step also monomeric and incorrect assembled oligomers of Hsp42 were separated. After this final purification step, Hsp42 was pooled, concentrated to ∼1 mg/ml by ultra filtration and stored at −80 °C for further use. Following this procedure, ∼0.3 mg Hsp42 per g *E. coli* wet weight, with more than 99% purity were obtained. In comparison to the purification of Hsp42 from *S. cerevisiae* [10] this corresponds to an increase of a factor of ∼10–15 in total amount of target protein per g cell mass.

We analyzed the CD spectrum of refolded Hsp42 to verify that it is structurally and functionally identical with the protein purified from yeast in its native, soluble form (Fig. 5A). The far UV spectrum of the refolded Hsp42 was nearly identical to the one purified from yeast and indicative of a protein containing α-helical segments (Fig. 5A). The low amplitude of the signal suggested a significant contribution of β-sheets. For several sHsps it was already demonstrated that the C-terminal α-crystallin domain consists of β-sheets [3,7,8,35–37], and the N-terminal parts were proposed to be unstructured or to include α-helical segments [3,4,7,8,37]. Using the deconvolution software CDNN (http://www.bioinformatik.biochemtech.unihalle.de/cdnn), a content of 31% α-helix and 18% β-sheet was calculated for Hsp42. For comparison, far UV spectra of Hsp26 [10,37] were indicative of a protein with a β-sheet content of 25% and a α-helical content of 23%. While CD spectra of both Hsp26 and Hsp42 show similar β-sheet contents, Hsp42 displayed a higher α-helical content. Thus, the calculated data are in agreement with structural data [35,36] indicating that for all sHsps the α-crystallin domain consists of β-sheets while the N-terminal parts of sHsps include α-helical and unstructured parts.

To verify the correct size of the Hsp42 oligomer, SEC of the refolded protein was performed (Fig. 5B). Like Hsp42 purified from yeast under native conditions, at a concentration of 1 mg/ml the refolded protein eluted as a single peak with an apparent molecular mass of 600–700 kDa (Fig. 5B).

To investigate the chaperone properties of refolded Hsp42, we analyzed the interactions of Hsp42 with Rhodanese. Like Hsp42 purified from yeast, the refolded protein suppressed the aggregation of Rhodanese completely at a molar ratio of 4:1 (Hsp42 monomer: Rhodanese monomer; Fig. 6A, data not shown).

To analyze whether refolded Hsp42 also forms stable complexes with Rhodanese, we incubated Rhodanese and Hsp42 at a molar ratio of 1:1 at 44 °C for 30 min and then applied the mixture to SEC. As shown in Fig. 6B, a new peak eluting in the void volume of the column could be detected. To verify that the new peak corresponds to a complex of Hsp42 and Rhodanese, the

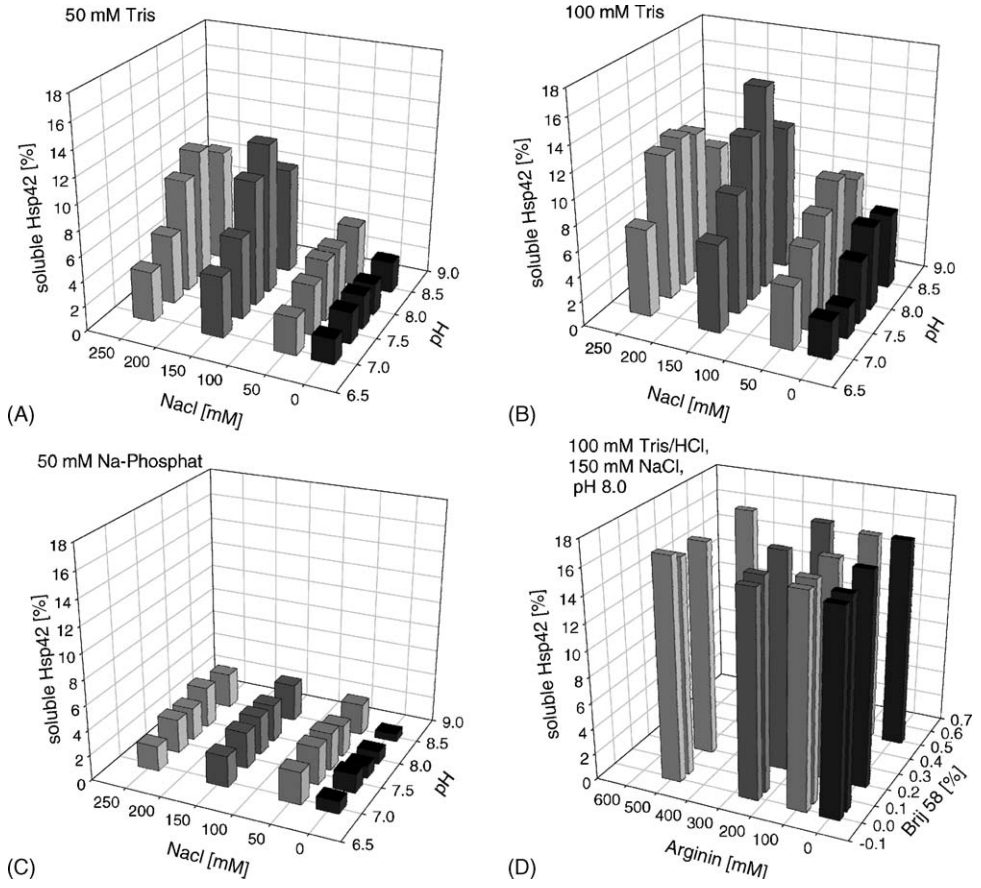

Fig. 3. Refolding screen of Hsp42. For refolding the protein was diluted into a subset of different refolding buffers. For single experiments 300 μg solubilized IB protein (concentration 6 mg/ml) was diluted to a final concentration of 30 μg/ml and incubated at 10 °C for 16 h. The samples were centrifuged and the amount of Hsp42 in the supernatant was quantified by SDS-PAGE and immunoblot. The percentage of soluble Hsp42 in comparison to the total amount of Hsp42 in the 300 μg solubilized IB (100%) was plotted. All refolding buffers contained 1 mM DTT and 2 mM EDTA. (A) Refolding of Hsp42 in 50 mM Tris buffer with varying NaCl concentrations and pH as indicated. (B) 100 mM Tris was used as basic buffer system. (C) A 50 mM sodium phosphate buffer was used. (D) Refolding of Hsp42 in 50 mM Tris/HCl, 150 mM NaCl, pH 8.0 with varying arginine and Brij 58 concentrations as indicated.

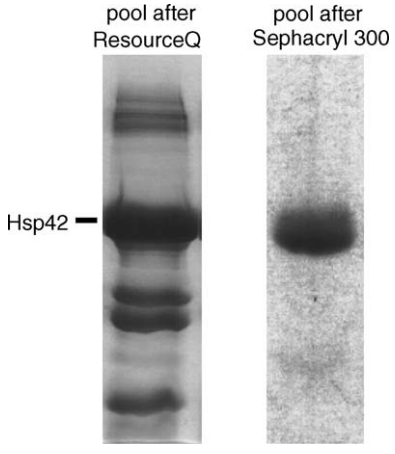

Fig. 4. Purification of Hsp42 after refolding. Coomassie stained SDS-PAGE comparing the Hsp42 pools after chromatography on a 6 ml Resource Q anion exchange column in 100 mM Tris/HCl, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, pH 8.0 using a gradient from 150 to 750 mM NaCl for elution (left) and after SEC on a 16/60 Sephacryl 300 pg column (Amersham Biosciences, Uppsala, Sweden) as final purification step (right).

peak was collected and applied to SDS-PAGE (Fig. 6B, *inset*). Both proteins were detected, indicating the formation of a complex. In a second approach, we analyzed complex formation of Hsp42 and Rhodanese by native-PAGE. We incubated Hsp42 and Rhodanese at a molar ratio of 4:1 for 30 min at 44 °C and separated the mixture by native-PAGE (Fig. 6C). A complex was detected, but also free Rhodanese and Hsp42 could be observed. Taken together, our experiments proved that the refolded Hsp42 was correctly assembled and fully functional.

The common view is that sHsps are energy independent chaperones, nevertheless, influences of ATP on the chaperone functions of αB-crystallin were described [38–41], suggesting structural rearrangements upon ATP binding [42,43]. To test whether ATP might influence the suppression of Rhodanese aggregation by Hsp42, we added increasing amounts of ATP to the protein mixture (Fig. 7A). However, no significant influence of ATP on the chaperone activity of Hsp42 was observed. In addition, no ATP hydrolysis by Hsp42 was observed even when very high amounts of Hsp42 (1–25 μg per assay) were used or if the temperature was varied between 10 and 45 °C (Fig. 7B; data not shown). This indicates that Hsp42 acts in an energy independent manner.

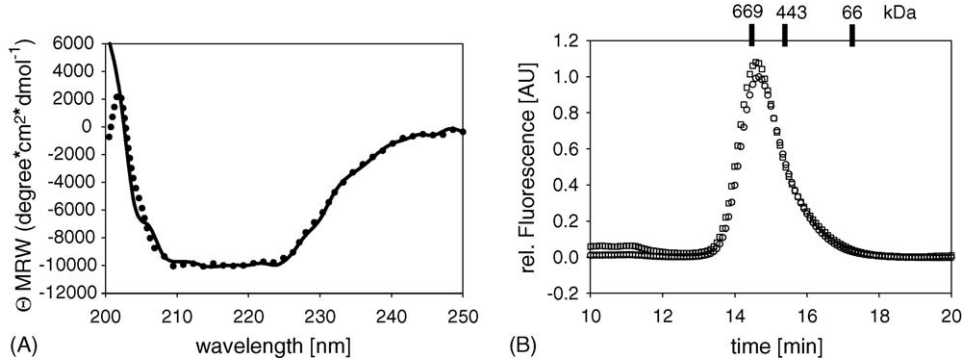

Fig. 5. The secondary and quaternary structure of refolded Hsp42 is identical to the native protein purified from the yeast cytosol. (A) Far-UV CD spectra of Hsp42. The spectra of the native protein purified from yeast (dotted line) and that of the refolded Hsp42 (solid line), were recorded in 50 mM potassium phosphate, pH 7.4. (B) Size exclusion chromatography. HPLC was performed using a TosoHaas TSK 4000 PW column as described in Section 2. Hsp42 concentrations were 1 mg/ml, refolded Hsp42 (◯) and native Hsp42 purified from yeast (□).

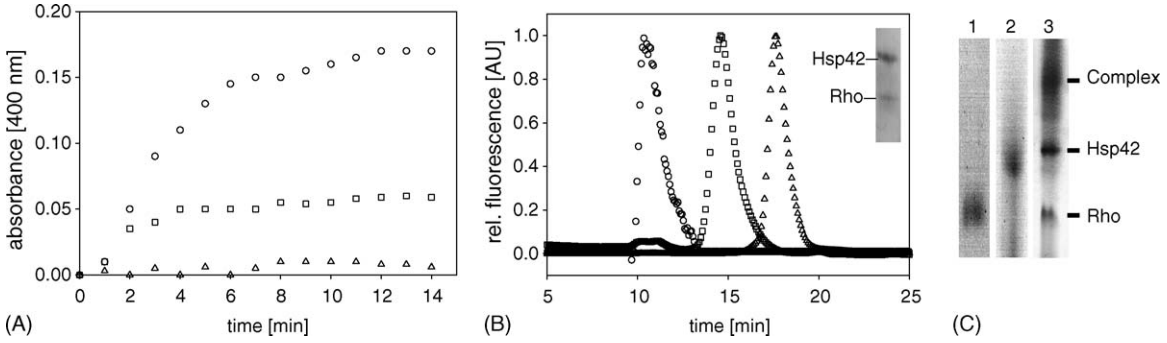

Fig. 6. Refolded Hsp42 suppresses the aggregation of Rhodanese and forms complexes at 44 °C. (A) Influence of refolded Hsp42 on the thermal aggregation of Rhodanese (Rho). Rhodanese (final concentration: 1 μM) was diluted into a thermostated solution of 2 μM (□), and 4 μM (△) Hsp42 monomer. Circles (◯) represent the spontaneous aggregation of Rhodanese at 44 °C. The kinetics of aggregation was determined by measuring the light scattering of the samples at 400 nm. (B) Complexes of Hsp42 and Rhodanese (◯) were analyzed after incubation of the proteins at a molar ratio of 1:1 (monomers) at 44 °C for 30 min. After incubation of the complex at 10 °C for 10 min, the complex was applied to SEC. For comparison, Rhodanese (△) and Hsp42 (□) alone were applied. Elution profiles were normalized. *Inset*, silver-stained SDS-PAGE of the collected complex peak. (C) Native-PAGE of complexes of Hsp42 and Rhodanese. Complexes of Rhodanese and Hsp42 at a molar ratio (Rhodanese monomer: Hsp42 monomer) of 1:4 were formed at 44 °C (lane 3). For comparison, Rhodanese (lane 1) and Hsp42 (lane 2) without incubation at 44 °C were separated.

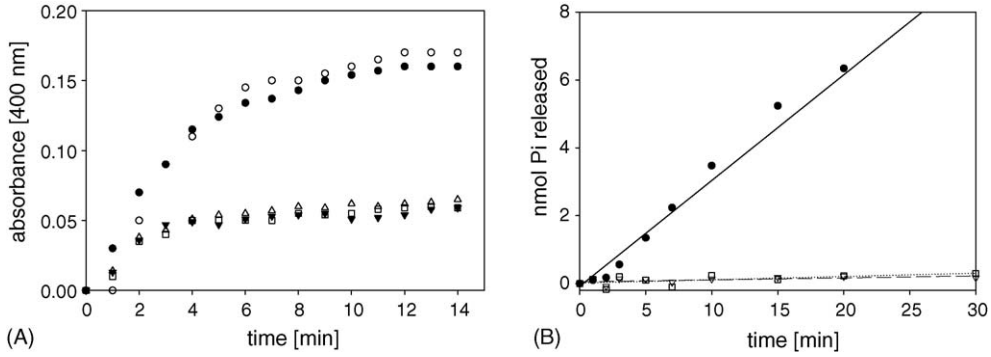

Fig. 7. ATP has no influence on the chaperone activity of Hsp42. (A) Influence of ATP on the suppression of thermal aggregation of Rhodanese (Rho) by Hsp42. Rhodanese (final concentration: 1 μM) was diluted into a thermostated solution of 2 μM of Hsp42 monomer in the absence of ATP (□) or in the presence of 3 mM (▼) or 6 mM ATP (△). Circles represent the spontaneous aggregation of Rhodanese at 44 °C in absence (◯) or presence (●) of 6 mM ATP. The kinetics of aggregation were determined by measuring the light scattering of the samples at 400 nm. (B) ATP hydrolysis was assayed following the change of Malachit Green absorbance at 660 nm in dependence of free phosphate [28]. ATP hydrolysis by 10 μg recombinant Hsp42 (□, dotted line) or 10 μg mature Hsp42 purified from yeast (◊, dashed line) in the presence of 5 mM ATP and 10 mM $MgCl_2$ at 37 °C. As a positive control the ATP hydrolysis by 1 μg of Hsp104 in the presence of 5 mM ATP and 10 mM $MgCl_2$ is shown (●, solid line). Activity values were calibrated against KH_2PO_4 standards and corrected for phosphate release in the absence of protein.

4. Discussion

Structural analysis of sHsps revealed that sHsps are dynamic oligomers [13–16,44–46]. Two different overall structural assemblies composed of dimeric building blocks have been described so far: globular, shell-like structures for α-crystallin, Hsp16.5 from *M. jannaschii* and Hsp26 from yeast [7,9,11] or double-ring structures for Hsp16.9 from wheat [8] and Hsp42 from yeast [10].

The sHsp system of *S. cerevisiae* consists of two components [10]. Together, they enable prevention of aggregation of cytosolic proteins under physiological and stress conditions. While, under physiological conditions, Hsp42 is already active and prevents unfolding intermediates from aggregation, the Hsp26 complex is an inactive storage form. Stress conditions, like heat shock, lead to the unfolding of a large number of cellular proteins. Concomitantly, Hsp26 is activated directly through temperature [9,16]; Hsp42 remains active as a chaperone at higher temperature. Both yeast sHsps are highly promiscuous and bind a broad variety of substrate proteins in vitro and in vivo [9,10]. In the case of Hsp42 this promiscuousness has the disadvantage that the purification of the protein is especially complicated due to the formation of stable Hsp42–substrate complexes during the purification procedure. To overcome this problem we synthesized Hsp42 in *E. coli*. Interestingly, Hsp42 proved to be insoluble, forcing us to isolate Hsp42 under denaturing conditions and subsequently refolding the protein. In this context, we demonstrated that thermal unfolding of Hsp42 was irreversible showing one structural transition with a midpoint at 49.5 °C [10]. However, after chemical unfolding Hsp42 was shown to refold efficiently in buffers with high ionic strength (150 mM NaCl). This observation is in good agreement with studies on Hsp26, where chemical unfolding also was completely reversible [37]. In addition the protein assembled to the correct oligomeric state and was fully active as a chaperone. The amounts of Hsp42 purified according to this strategy were 10–15 times higher compared to the isolation of native Hsp42 from yeast.

A remaining controversy is the influence of ATP on the chaperone activity of sHsps [38–40,42,43]. Generally sHsps are believed to act in an energy independent manner [3–5,47]. The addition of ATP did not enhance the chaperone activity of *E. coli* IbpB [48], *Mycobacterimu tuberculosis* Hsp16.3 [49], murine Hsp25 [19] or pea sHsps [50]. On the other hand, modulation of the chaperone properties of α-crystallin has been proposed, where ATP induced a conformational change that was accompanied by a concomitant internalization of a previously exposed hydrophobic surface [51]. In this context four conserved residues in the core domain of α-crystallin seem to become protected against proteolytic cleavage in the presence of ATP. This may indicate ATP-dependent structural modifications and an ATP-binding motif in the core domain of sHsps [39]. This structural rearrangement might lead to an ATP-enhanced chaperone activity of α-crystallin as monitored by a two-fold increase in suppression of CS aggregation in presence of ATP.

Here, we demonstrate that the chaperone properties of Hsp42 are also not influenced by ATP. In addition, no hydrolysis of ATP by Hsp42 could be detected indicating that it acts in an ATP independent manner. In summary, this observation strengthens the hypothesis that ATP dependence might be specific for α-crystallin [3,38]. In the eye lens, the ATP concentration is significantly higher than in most other tissues making it highly plausible that in the lens, stimulation of α-crystallin might be crucial for optimal chaperone activity.

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