Oligomerization, Chaperone Activity and Nuclear Localization of p26, a Small Heat Shock Protein from *Artemia franciscana*

Sun, Yu, Mansour, Marc, Crack, Julie A., Gass, Gillian L., MacRae, Thomas H.

*Department of Biology, Dalhousie University, Halifax, Nova Scotia, B3H 4J1, Canada*

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Author for Correspondence:

Thomas H. MacRae

Department of Biology

Dalhousie University

Halifax, N.S. B3H 4J1

Canada

Telephone: 902-494-6525

FAX: 902-494-3736

E-mail: tmacrae@dal.ca
Abstract: *Artemia franciscana* embryos undergo encystment, developmental arrest and diapause, the latter characterized by profound metabolic dormancy and extreme stress resistance. Encysted embryos contain an abundant small heat shock protein termed p26, a molecular chaperone that undoubtedly has an important role in development. To better understand the role of p26 in *Artemia* embryos, the structural and functional characteristics of full length and truncated p26 expressed in *Escherichia coli* and COS-1 cells were determined. p26 chaperone activity declined with increasing truncation of the protein, and those deletions with the greatest adverse effect on protection of citrate synthase during thermal stress had the most influence on oligomerization. When produced in either prokaryotic or eukaryotic cells the p26 α-crystallin domain consisting of amino acid residues 61-152 existed predominately as monomers, and p26 variants lacking the amino terminal domain but with intact carboxy-terminal extensions were mainly monomers and dimers. The amino-terminus was, therefore, required for efficient dimer formation. Assembly of higher order oligomers was enhanced by the carboxy-terminal extension, although removing the ten carboxy-terminal residues had relatively little effect on oligomerization and chaperoning. Full length and carboxy-terminal truncated p26 resided in the cytoplasm of transfected COS-1 cells, however variants missing the complete amino-terminal domain and existing predominantly as monomers/dimers entered nuclei. A mechanism whereby oligomer disassembly assisted entry of p26 into nuclei was suggested, this of importance because p26 translocates into *Artemia* embryo nuclei during development and stress. However, when examined in *Artemia*, p26 oligomer size was unchanged under conditions that allowed
movement into nuclei, suggesting a process more complex than just oligomer dissociation.

INTRODUCTION

The small heat shock proteins (sHSPs), characterized by a conserved α-crystallin domain exhibiting an immunoglobulin-like fold bordered by variable amino- and carboxy-terminal extensions (1-7), function as molecular chaperones. The molecular mass of sHSPs ranges from 12-43 kDa and most oligomerize by a multi-step process often with dimers as stable sub-oligomeric units (8, 9), although for bovine αB-crystallin monomers may be basic building blocks (10). For sHSPs such as HSP20 (11) and HSP22 (12), dimers are the predominant complex and they exhibit chaperone activity. Of medical significance, αA-crystallin truncations occur in mammalian lens, suggesting a relationship between carboxy-terminal modification and cataract (13, 14). Also, dropping either thirteen or twenty-five carboxy-terminal residues from human αB-crystallin causes myofibrillar myopathy, with modified proteins exerting dominant negative effects (15). The truncated and normal αB-crystallins appear to interact, yielding oligomers slightly smaller than those generated by wild type protein.

Assembly mechanisms and the resulting oligomers vary for sHSPs from different sources (2, 4, 16-18). For example, oligomers of HSP16.5 from the archaea, Methanococcus jannaschii (19) and HSP16.9 from wheat, Triticum aestivum (18), the only crystallized sHSPs, are monodisperse, demonstrating well defined stoichiometry. In contrast, oligomers from other
species are polydisperse and resist crystallization (3, 10, 20). sHSP oligomers exhibit rapid subunit exchange, a property that influences chaperone function (9, 11, 16, 21-25). The sHSPs bind proteins in the molten globular state which are primed for aggregation and potential irreversible precipitation (26). These substrates, involved in functions from transcription to secondary metabolism, are subsequently released and refolded, activities reported to depend upon ATP-requiring chaperones such as HSP70 (1, 21, 27-30).

Embryos of the brine shrimp, *Artemia franciscana*, undergo ovoviviparous and oviparous development, the former yielding nauplii and the latter encysted gastrulae or cysts (31). Cysts enter diapause (32, 33), characterized by deep reduction in metabolic activity (34) and resistance to extreme environmental stress such as long term anoxia, desiccation and heat shock (35, 36). p26, an abundant sHSP in *Artemia* cysts, forms oligomers, functions as a molecular chaperone *in vitro* and confers thermotolerance on transformed bacteria (29, 37-41), undoubtedly contributing to embryo stress tolerance. p26 enters nuclei upon synthesis in developing embryos (38), and migrates reversibly by a poorly understood mechanism into *Artemia* nuclei during anoxia and heat shock (42-44). Upon exposure to anoxia the internal pH of post-diapause cysts drops to about 6.6, and reversible movement of p26 into nuclei occurs *in vitro* upon pH reduction. Domain-specific effects on p26 oligomerization, chaperone activity and nuclear localization were examined in this study and the data are related to the structural/functional properties of p26 within oviparously developing *Artemia* embryos.
EXPERIMENTAL PROCEDURES

Cloning of p26 cDNAs-Truncated p26 cDNAs were generated by site-directed mutagenesis using the QuikChange\textsuperscript{TM} Site-directed Mutagenesis kit (Stratagene, La Jolla, CA), p26-3-6-3 as template (38), and designated primers (Table 1). The truncated p26-3-6-3 cDNAs were recovered from agarose gels with the GFX\textsuperscript{TM} PCR DNA and Gel Band purification kit (Amersham Biosciences, Piscataway, NJ), inserted into pRSET.C, a polyhistidine-tagged (His-tagged) prokaryotic expression vector linearized by digestion with BamH1 and Xho1 prior to purification from agarose gels, and cloned in *Escherichia coli* strain BL21(DE3)PlysS (Invitrogen, Carlsbad, CA). Full-length and truncated p26 cDNAs were also generated previously by PCR and inserted into the T/A vector, pCRII (37). The p26 cDNA-containing pCRII vectors and the mammalian expression vector pSecCMV (Invitrogen) were digested with *BamH1* and *Xba1*, followed by electrophoresis in 1% agarose gels. p26 cDNAs were recovered with the GFX\textsuperscript{TM} PCR DNA and Gel Band purification kit, inserted into linearized pSecCMV, and cloned in *E. coli* DH5\textalpha\textsuperscript{TM} (Invitrogen). Full length p26 cDNA (p26-full) and the cDNA fragments p26-N60 and p26-alpha were excised from pSecCMV with *BamH1* and *Xba1*, inserted into pcDNA4/His.A (Invitrogen), a His-tag-containing mammalian expression vector, and cloned in *E. coli* DH5\textalpha. All inserts were sized by restriction digestion followed by electrophoresis in agarose, and PCR fidelity was verified by DNA sequencing (DNA Sequencing Facility, Centre for Applied Genomics, Hospital for Sick Children, Toronto, ON).
Purification of p26-Expression plasmids containing either full length or truncated p26 cDNAs transformed into *E. coli* strain BL21(DE3)PLysS were induced by addition of 1 mM isopropyl thio-β-D-galactoside (IPTG) (Sigma, St. Louis, MO) for 5 h. The cultures were cooled on ice and centrifuged at 5,000 × g for 5 min at 4 °C. The cell pellets were washed once with Extraction/Wash buffer (50 mM Na₃PO₄, 300 mM NaCl, pH 7.5) and resuspended in 4 ml of the same buffer prior to addition of 100 µg/ml lysozyme (Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma), 1 µg/ml pepstatin A (Sigma) and 1 µg/ml leupeptin (Sigma). The mixtures were incubated at 30 °C for 15 min, cooled on ice and sonicated three times for 10 sec using a Branson Sonifier™ 150 (Branson Ultrasonics, Danbury, CT) at medium setting with intermittent cooling on ice for 30 sec. The sonicated cells were centrifuged at 12,000 × g for 20 min at 4 °C, the supernatants recovered and protein concentrations ascertained with the Bio-Rad Protein determination kit (Bio-Rad, Hercules, CA). His-tagged p26 was purified from bacterial extracts using 2 ml BD TALON™ affinity columns as described by the manufacturer (BD Biosciences, Mississauga, ON). The samples were desalted by dialysis against 10 mM NaH₂PO₄, pH 7.1 and concentrated with Centriprep YM-10 centrifugal devices (Amicon Bioseparations, Billerica, MA).

Bacterial extracts and purified p26 were electrophoresed in 12.5% SDS polyacrylamide gels followed either by staining with Coomassie blue or transfer to nitrocellulose. Blots were stained with 2% Ponceau S (Sigma) in 3% trichloroacetic acid (TCA) to verify protein transfer, washed
with TBS-Tween (10 mM Tris-HCl, 140 mM NaCl, 0.1% (v/v) Tween 20, pH 7.4) and incubated for 45 min with shaking in 5% low fat Carnation milk powder in TBS-Tween. The blots were incubated with Omni-probe (Santa Cruz Biotechnology, Santa Cruz, CA), an anti-His antibody diluted in TBS-Tween, followed by goat anti-mouse IgG HRP-conjugated antibody (Jackson ImmunoResearch, Mississauga, ON) diluted in TBS-Tween. Protein detection was conducted with the Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA).

Oligomer formation by purified p26- Half ml samples of full length and truncated p26 proteins purified from *E. coli* were applied to continuous 10-50% (w/v) sucrose gradients and centrifuged at 200,000 × g for 21 h at 4 °C in a Beckman SW41 Ti rotor. Tube bottoms were punctured, 0.8 ml fractions collected and the A_{280} of each sample measured with a SPECTRAMax PLUS microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The number of p26 monomers per oligomer was calculated using a p26 molecular mass of 20.8 kDa as determined by GENERUNNER with corrections for addition and deletion of amino acid residues as necessary. Alpha-lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), apoferritin (443 kDa), and thyroglobulin (669 kDa) (Sigma) were centrifuged separately and their locations determined by measuring the A_{280} of gradient fractions.

Chaperone activity of p26-A 178 mM stock solution of dimeric citrate synthase (Sigma) was
diluted with 40 mM HEPES/KOH buffer at pH 7.5 to a final concentration of 150 nM in 1.0 ml cuvettes and heated at 43 °C with 37.5, 75, 150, 300 and 600 nM of purified, bacterially-produced p26. p26 molarity in reaction mixtures was based on monomeric molecular masses of p26 variants. Citrate synthase aggregation was monitored by measuring solution turbidity at 360 nm with a SPECTRAmax PLUS spectrophotometer every 2 min for 1 h. Bovine serum albumin (BSA) (Sigma) and immunoglobulin G (IgG) (Sigma) were used at 600 nM to evaluate non-specific protection of citrate synthase.

\[ p26 \text{ expression in transiently transfected COS-1 cells} \]

COS-1 cells were maintained at 37 °C under 5% CO\(_2\) in Dulbecco’s Modified Eagles Medium (DMEM) (Gibco, Burlington, ON) containing 10% fetal bovine serum (Gibco) and 2% penicillin/streptomycin (Gibco). Cells in single T75 culture flasks (Becton Dickenson, Franklin Lakes, NJ) were transfected with mixtures containing 60 µl of DMEM lacking serum and antibiotics, 1 µg of plasmid DNA and 3 µl of SuperFect™ (Qiagen, Mississauga, ON). Prior to use, transfection mixtures were incubated at room temperature for 15 min, then placed in T75 flasks containing cells grown to 60% confluency and incubated at 37 °C. The cells were trypsinized 24 h after transfection, collected by centrifugation at 1,500 \( \times \) g for 5 min, washed with 1 ml of PBS, centrifuged at 1,500 \( \times \) g for 5 min, incubated on ice for 20 min in 50 µl of SDS polyacrylamide gel treatment buffer, and centrifuged at 10,000 \( \times \) g for 10 min. Supernatant protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA). Equal volumes of cell-free extract were electrophoresed
in 12.5% SDS polyacrylamide gels and either stained with Coomassie blue or transferred to nitrocellulose membranes. Immunoprobing of membranes was with Omni-probe as described earlier, or with antibody to p26 (39) followed by HRP-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch).

\(p26\) oligomer formation in COS-1 cells-Transfected COS-1 cells grown to confluency in T75 culture flasks and collected as described above were incubated on ice for 20 min in 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, pH 7.8 containing 1 mM PMSF, 1 µg/ml pepstatin A and 1 µg/ml leupeptin, before centrifugation at 10,000 \(\times\) g for 10 min. Equal amounts of protein extract from transfected COS-1 cells were applied to individual 10-50% sucrose gradients, prepared by layering 5 ml of 10% (w/v) sucrose on 5 ml of 50% sucrose in 0.1 M Tris/glycine buffer, pH 7.4, in 12 ml tubes and centrifuging at 200,000 \(\times\) g for 3 h at 15 °C. The gradients were centrifuged at 200,000 \(\times\) g for 21 h at 4 °C followed by collection of 0.8 ml fractions. Fifteen µl from each fraction was electrophoresed in 12.5% SDS polyacrylamide gels, blotted to nitrocellulose, and probed with either anti-p26 antibody or Omni-probe as described previously.

Immunolocalization of \(p26\) in transfected COS-1 cells-Sixty µl of DMEM without serum or antibiotics, 1 µg of plasmid DNA and 3 µl of SuperFect™ were mixed for individual wells of 6-well Falcon culture plates (Becton Dickenson), incubated at room temperature for 15 min, placed on cells grown to 60% confluency on cover-slips in 6-well plates, and incubated at 37 °C for 24 h. Cover-slips were then placed in humidity chambers, rinsed with phosphate buffered saline.
(PBS) (140 mM NaCl, 2.7 mM KCl, 8.0 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, pH 7.4), and fixed for 20 min at room temperature in 4% paraformaldehyde. The cells were rinsed twice with PBS, permeabilized 5 min in PBS containing 0.2% (v/v) Triton X-100 (PBS-Triton) and incubated at room temperature for 30 min with anti-p26 antibody (39) diluted in PBS-Triton. The cells were washed three times with PBS-Triton then incubated for 20 min at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch) diluted in PBS-Triton. After washing three times with PBS-Triton, the cells were incubated with RNaseA (Amersham Biosciences, Baie d’Urfe, QC) at 5 mg/ml for 10 min and stained with propidium iodide (Sigma) at 0.4 mg/ml for 2 min. Cover-slips were rinsed with distilled water and placed on Vectashield™ mounting medium (Vector Laboratories, Burlingame, CA). Cells expressing His-tagged p26 fusion proteins were also stained with Omni-probe diluted in PBS-Triton, followed by FITC-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch). Immunofluorescently stained cells were examined with a Zeiss 410 confocal laser scanning microscope.

Effect of pH on p26 oligomers-Encysted Artemia embryos (cysts) (INVE Aquaculture, Inc., Ogden, UT) were hydrated in distilled water at 4 °C for 3 h, collected on a Buchner funnel, washed with cold distilled water and homogenized with a Retsch motorized mortar and pestle (Brinkman Instruments Canada, Rexdale, ON) in Pipes buffer (100 mM Pipes, 1 mM EGTA, 1 mM MgCl$_2$) at either pH 6.5 or 7.0. The homogenates were centrifuged at 16,000 x g for 10 min
at 4 °C and the supernatants were passed through two layers of Miracloth (Calbiochem, La Jolla, CA) before centrifugation at 40,000 x g for 30 min at 4 °C. The upper two thirds of each supernatant was transferred to a fresh tube and re-centrifuged. Either immediately after preparation, or following incubation at room temperature for 30 min, supernatants were centrifuged at 200,000 x g for 12 h at 4 °C in 10-50% continuous sucrose gradients at the pH used for homogenization. The gradients were fractionated and samples were electrophoresed in SDS polyacrylamide gels, blotted to nitrocellulose and probed with anti-p26 antibody.

*Effect of heat shock on p26 oligomers*- Ten g of *Artemia* cysts in 500 ml of distilled H2O were brought to 22 °C, then heated to 50 °C over 1 h with vigorous aeration in a Programmable/Digital Immersion Circulator (VWR, Mississauga, ON). Heat shocked and control cysts, the latter incubated on ice for 30 min, were homogenized separately by hand for 2 min in a chilled mortar and pestle in 35 ml of cold HPC (1 mM CaCl2, 0.05 mM PIPES, 6.4% Hexylene glycol, pH 7.0), followed by one passage in a Dounce homogenizer, filtration through Miracloth, and centrifugation at 2,000 x g for 10 min at 4 °C to pellet nuclei. Supernatants were centrifuged at 40,000 x g for 30 min at 4 °C, and the upper two thirds was placed in a fresh tube and centrifuged. Supernatants were centrifuged in 10-50% continuous sucrose gradients, fractionated and analyzed on western blots after SDS polyacrylamide gel electrophoresis.

The nuclei-containing pellets from above were rinsed twice with 40 ml of HPC, resuspended
in 17.5 ml of the same buffer, applied to 25 ml cushions of 75% (v/v) Percoll (Sigma) in 150 mM NaCl, 14 mM MgCl₂, 16 mM Tris, pH 7.0, and centrifuged at 16,000 \( \times g \) for 30 min at 4°C in a Beckman JS-13.1 swinging bucket rotor. Cloudy layers were transferred to fresh tubes, brought to 15 ml with HPC, placed on a 25 ml Percoll cushion and centrifuged as above. Nuclei were recovered, mixed with an equal volume of HPC, centrifuged at 16,000 \( \times g \) for 30 min at 4°C, and the nuclei-containing pellets were suspended in 1.0 ml of HPC. Samples were stained with 0.4% DAPI (Molecular Probes, Eugene, OR) and examined microscopically. Nuclei from control and heat shocked embryos were lysed in treatment buffer, electrophoresed in 12.5% SDS polyacrylamide gels, blotted to nitrocellulose and probed with anti-p26 antibody to determine if p26 had moved into nuclei. For sucrose density gradient centrifugation, extracts were prepared by suspending purified nuclei in 1.0 ml of ice cold extraction buffer (20 mM HEPES-KOH, 2.5 mM MgCl₂, 0.2 M NaCl, 25% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, pH 7.9) containing 1 mM PMSF, 1 µg/ml pepstatin A and 1 µg/ml leupeptin, and sonicating three times for 10 sec using a Branson Sonifier™ 150 at medium setting with intermittent cooling on ice for 30 sec. The sonicated samples were incubated on ice for 30 min, and then centrifuged at 25,000 \( \times g \) for 30 min at 4°C. Nuclear extracts were centrifuged in 10-50% continuous sucrose gradients at 200,000 \( \times g \) for 12 h at 4°C. The gradients were fractionated and samples were electrophoresed in SDS polyacrylamide gels, blotted to nitrocellulose and probed with antibody
to localize p26.

RESULTS

Cloning of p26 cDNAs—Full length and truncated p26 cDNAs cloned in the prokaryotic expression vector pRSET.C included p26-full-His, the full length polypeptide; p26-NΔ36-His, residues 1-36 deleted; p26-NΔ60-His, residues 1-60 removed; p26-CΔ40-His, final forty residues eliminated; p26-CΔ10-His, lacked the last ten residues; p26-alpha-His, missing residues 1-60 and 153-192, thereby consisting of the α-crystallin domain (Fig. 1). All p26 cDNAs generated by site-directed mutagenesis contained, in addition to the p26 sequence, an amino terminal peptide of 13 residues encoded by p26-3-6-3 and the His-tag. The presence of the His-tag is indicated by “-His” in the name of the p26 cDNA or protein. The p26 cDNAs recovered from the storage vector pCRII (37) were cloned in pSecCMV for expression in COS-1 cells. p26-full, p26-NΔ60 and p26-alpha were also prepared in pcDNA4/His.A for expression as His-tagged fusion proteins. All cloned p26 cDNA fragments were sized by electrophoresis in agarose after restriction digestion of recombinant plasmids and PCR fidelity was confirmed by sequencing all cloned inserts (not shown).

p26 oligomer formation and chaperone activity—IPTG induction of transformed E. coli yielded polypeptides on western blots of the appropriate size that reacted strongly with Omni-probe, although bands corresponding to p26 were not readily visible in Coomassie blue stained gels (Figs. 2a, b). With the exception of p26-NΔ60-His and p26-alpha-His, all of the polypeptides
were recognized by antibody to p26 (not shown). Subsequent to purification on TALON™ affinity columns single bands of the expected size appeared on Coomassie blue stained gels (Fig. 2c). These polypeptides reacted strongly with Omni-probe (Fig. 2d) indicating they were His-tagged p26, although purified p26-NΔ60-His and p26-alpha-His were again not recognized by anti-p26 antibody. Protein bands were not detected on blots containing extract from E. coli transformed with vector lacking p26 cDNA (Figs. 2a, b, lane 7).

The molecular mass of oligomers assembled from purified p26-full-His synthesized in E. coli ranged from 26 kDa to 600 kDa, with the peak at about 150 kDa, or 6 monomers per oligomer (Fig. 3, Table 2). The oligomers assembled with full length p26 were larger than those obtained with truncated proteins, although oligomers of p26-CΔ10-His were similar in mass. At the other extreme, p26-alpha-His migrated predominately as monomers and p26-NΔ60-His existed as monomers and dimers, although larger complexes occurred. Oligomers assembled from other p26 variants were intermediate in mass to those just described.

Purified p26-full-His possessed the greatest chaperone activity and p26-alpha-His the least, as determined by heat-induced denaturation of citrate synthase, although all variants provided some protection (Fig. 4). At a chaperone to target molar ratio of 4:1 (p26 monomer to citrate synthase dimer), or 1:1.5 if the peak oligomer size of p26-full-His is used for comparison, citrate synthase protection was almost complete after 1 h at 43 °C (Fig. 4a). At a 1:4 molar ratio of p26-full-His to citrate synthase, or 1:24 (oligomer to dimer), purified p26 reduced the heat-
induced turbidity increase by approximately 25% (Fig. 4e). In contrast, p26-alpha-His was almost devoid of chaperone activity even at molar ratios of 4:1 (Fig. 4). p26-N\(\Delta\)60-His was marginally better than p26-alpha-His as a chaperone, followed by p26-N\(\Delta\)36-His and p26-C\(\Delta\)40-His which were similar to one another. p26-C\(\Delta\)10-His approached p26-full-His in chaperone potency especially at high concentrations. BSA and IgG at 600 nM provided almost no protection upon heating of citrate synthase (Fig. 4f).

Synthesis and oligomerization of p26 in transfected mammalian cells-Except for p26-N\(\Delta\)60 and p26-alpha, which reacted with Omni-probe, protein extracts from COS-1 cells transfected with p26 cDNA-containing expression vectors yielded polypeptides of the expected size on western blots with anti-p26 antibody (Fig. 5). Neither primary antibody gave protein bands on blots with extracts from COS-1 cells transfected with vectors lacking p26 cDNA (not shown). Full length p26 synthesized in COS-1 cells produced oligomers as large as 512 kDa and composed of up to 21 monomers, with monomer number essentially the same in the presence and absence of His-tag (Figs. 6a, b). Oligomers assembled with p26-C\(\Delta\)10 were only slightly smaller than those produced from full length p26 (Fig. 6c), but removal of the complete carboxy-terminal extension gave oligomers of a narrower size range and smaller mass (Fig. 6d). Oligomer mass became progressively smaller upon sequential removal of the amino-terminus (Figs. 6e, f) with polypeptides encoded by p26-N\(\Delta\)60-His present as monomers and dimers, as was true for p26-alpha-His (Fig. 6g), although the latter was more enriched in monomers. The properties of p26 oligomers produced in COS-1 cells are summarized in Table 3.
Truncated p26 localizes to COS-1 nuclei-In order to monitor p26 synthesis transiently transfected COS-1 cells were stained with anti-p26 antibody, Omni-probe and propidium iodide, revealing that polypeptides encoded by p26-full and p26-full-His (Figs. 7a, b) and the carboxy-terminal truncations, p26-CΔ40 (Fig. 7c) and p26-CΔ10 (Fig. 7d), localized exclusively to the cytoplasm. In contrast, upon amino-terminal modification p26 occurred in cytoplasm and nuclei, the latter shown by yellow staining. p26-NΔ36 encoded polypeptides were in the nuclei of only some cells (Figs. 7e, f), but polypeptides p26-NΔ60/p26-NΔ60-His lacking the amino-terminus (Figs. 7g, h), or p26-alpha/p26-alpha-His composed of the α-crystallin domain (Figs. 7i, j), resided in the nuclei of all transfected cells, indicating that the His-tag had no effect on p26 localization. The results suggested that disassembly of oligomers is responsible for p26 movement into nuclei of COS-1 cells and by extrapolation, the nuclei of encysted Artemia embryos.

Oligomer size of p26 from Artemia embryos is unaffected by pH and heat shock-p26 moves into the nuclei of Artemia embryos upon exposure to reduced pH in vitro and upon heat shock in vivo by an unknown mechanism that, as just suggested by the previous results, entails oligomer mass reduction. However, the p26 oligomers obtained from Artemia cysts homogenized at either pH 6.5 or 7.0 were identical in molecular mass, whether or not they were incubated 30 min at room temperature before gradient centrifugation (Fig. 8a-d). p26 migrated into the nuclei of heat shocked Artemia embryos under the conditions used in this work (Figs. 9a, b), but the size of p26 oligomers in extracts from cysts (Figs. 9c, d) and nuclei (Figs. 9e, f) remained constant.
The p26 in nuclear extracts tended to smear upon electrophoresis and blotting, probably due to the presence of DNA.

DISCUSSION

Full length p26 produced in either bacteria or mammalian cells yielded oligomers that were somewhat smaller than those from encysted Artemia embryos, but which represented effective oligomerization nonetheless. In contrast, the α-crystallin domain existed mainly as monomers and dimers, as was true for p26 lacking the entire amino-terminus. These data indicate a role for the amino-terminus in formation of dimers and thus higher oligomers, a conclusion strengthened by the comparatively greater oligomerization of p26 lacking thirty-six rather than sixty residues. The α-crystallin domain and amino-terminal truncated p26 variants were poor chaperones in relation to full length p26, with chaperone activity decreasing as truncation increased. Reduced chaperone activity and oligomerization were likely caused by the loss of residues involved in oligomer formation and interaction with substrates. As one example, the p26 motif 17WSDPF21 corresponds to 15SWEPF19 in Chinese hamster HSP27, a sequence important for oligomer formation and chaperoning (45). p26 structural organization and function may also depend on other hydrophobic elements of the amino-terminal region with the deleted residues 1-36 and 1-60 possessing 72.2% and 61.7% hydrophobicity, respectively, both significantly higher than the 51% hydrophobicity of the full length protein.

In comparison to p26, yeast HSP26 lacking amino-terminal sequences formed dimers devoid
of chaperone activity even though dissociation of oligomers into dimers is a functional prerequisite (8). Human αB-crystallin missing amino acid residues 1-56 produced dimers with significant chaperone activity (46), while removing either 50 or 56 residues from the amino-terminus of αA-crystallin decreased oligomer mass from 550 to 60 kDa, the latter composed of tetramers and dimers (47). The results indicate that sHSP dimerization occurs independent of the amino-terminal domain, but formation of larger oligomers requires this region, conclusions in partial agreement with the findings for p26. HSP16.5 from M. jannaschii (19) and HSP16.9 from T. aestivum (18) assemble well defined (monodisperse) oligomers and for HSP16.5 the amino terminus facilitates oligomerization, but does not necessarily determine the final architectural structure (48). Deletion of 42 amino-terminal residues from rice HSP16.9 decreased chaperone activity but not oligomer size (49), and in the bacterium Bradyrhizobium japonicum, where the amino-terminus is required for oligomer assembly, at least a portion of the region drives dimer formation (17), as appears to be true for p26.

Removal of ten carboxy-terminal residues had little effect on p26, however deleting the entire carboxy-terminus, including the conserved motif I/V-X-I/V (as VPI) (17), reduced oligomerization and chaperoning. The carboxy-terminus of p26 was not required for dimer formation, but the region contributed to oligomer assembly, although less so than the amino-terminus. Deletion of the entire p26 carboxy-terminal extension reduced protection of citrate synthase upon heating, indicating a role in chaperoning which may depend upon oligomerization, recognition of substrate proteins, chaperone/substrate solubility, or a combination of these.
Precipitation of truncated p26 was not observed even with complete removal of the carboxy-terminus which contains twenty polar and four charged amino acids in the final thirty residues. In contrast, loss of the last sixteen amino acids from *C. elegans* HSP16-2 was without effect on oligomer size and chaperone activity, but the modified protein precipitated upon freeze/thawing (50), suggesting reduced solubility.

The carboxy-terminus promotes sHSP/substrate solubility and chaperoning to varying degrees (17, 50-53). Eliminating the last eighteen amino acid residues from mouse HSP25, which excludes the conserved I/V-X-I/V motif (17), had little affect on oligomerization and chaperoning of citrate synthase at 43 °C, but protection of $\alpha$-lactalbumin against dithiothreitol-induced denaturation was lost (52). Deletion of 10 carboxy-terminal residues has minimal impact on $\alpha$A-crystallin, and sometimes even enhanced substrate protection, albeit modestly (14, 47), but this result was never obtained with p26. In contrast, removal of 11 or more C-terminal residues from $\alpha$A-crystallin, including the I/V-X-I/V motif drastically reduced oligomer size and chaperoning, with Arg-163 particularly important. Additionally, the carboxy-terminus of the *B. japonicum* sHSP, and especially the conserved motif I/V-X-I/V, plays a role in oligomer assembly (17), as is true for Pfu-sHSP from the hyperthermophilic microorganism *Pyrococcus furiosus* (54), all results in agreement with those obtained with p26.

The role of sHSPs in nuclei has received limited attention. $\alpha$B-crystallin and HSP27 associate with nuclear speckles and nucleoli of various human cell lines in non-stress conditions.
and may exert regulatory roles in these locations (55, 56). Human HSP27 translocates into nuclei of transfected A549 cells occurs stress, although protection occurs independent of nuclear localization (57). p26 migrates into nuclei early in oviparous development (38), during physiological stress and upon exposure to acidic conditions in vitro (42-44). A nuclear localization signal as occurs in tomato HSP16.1-CIII (58) is not apparent, but residues 36-45 of p26 include six arginines (39), a potential nuclear localization signal.

In this study, full length and carboxy truncated p26 resided exclusively in transfected COS-1 cell cytoplasm. In contrast, complete removal of the amino-terminal domain, including the putative nuclear localization signal, resulted in nuclear translocation. One interpretation of this finding is that decreased oligomer disassembly permits p26 movement through nuclear pores by simple diffusion, and nuclear translocation of rat HSP20 and HSP25 upon heat shock, where they may play protective roles, is accompanied by stress-induced decrease in oligomer mass, perhaps to dimers for HSP20 (59). However, p26 reduced in oligomer size due to carboxy-terminal truncation remained in the cytoplasm, suggesting that simple diffusion is not occurring. In support of this, modification of a single p26 residue by site-directed mutagenesis had little effect on oligomerization, but led to nuclear localization (manuscript in preparation). Additionally, p26 oligomers in heat stressed and control cysts, including those from nuclei, were similar and their mass was maintained in reduced pH, a condition that promotes p26 translocation into nuclei in vivo and in vitro. The most direct conclusion is that p26 migration into cyst nuclei depends on a mechanism other than oligomer mass reduction, although the transient formation of monomers or
dimers followed by reassembly in the nucleus is possible. Clearly, however, movement of p26 into the nuclei occurs independent of the arginine-enriched amino-terminal sequence.

To conclude, the amino-terminal domain of p26, a sHSP occurring in embryos that exhibit extreme stress resistance, promotes \(\alpha\)-crystallin dimerization and in concert with the carboxy-terminal extension enhances protein oligomerization. As measured by heat-induced denaturation of citrate synthase, the \(\alpha\)-crystallin domain of p26 lacks effective chaperone activity, depending on amino- and carboxy-terminal regions for full function. The nuclear translocation of p26 in COS-1 cells occurs independent of oligomer size and a putative nuclear localization in the amino-terminal region, suggesting a complex mechanism for movement into the nuclei of encysted *Artemia* embryos. Additional work to support these conclusions and to define domain-specific p26 amino acid residues with structural/functional implications is underway.

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FIGURE LEGENDS

Fig. 1. Schematic representation and sequence of p26 cDNAs. a, p26 cDNAs generated by site-directed mutagenesis and PCR were cloned in the prokaryotic expression vector pRSET.C and the eukaryotic expression vectors pSecCMV and pcDNA4/His.A. The primers used for site-directed mutagenesis are listed in Table 1. Box labeled 6xHis, His-tag-encoding sequence present in the vectors pRSET.C and pcDNA4/His.A. b, Amino acid sequence of p26 (Accession Number: AF031367). The amino acid residues removed to generate p26-NΔ60 and p26-CΔ40 are shaded and those for p26-NΔ36 and p26-CΔ10 are shaded and bold.
Fig. 2. **Purification of p26 synthesized in* E. coli*. Cell free extracts from transformed *E. coli* induced with IPTG were electrophoresed in SDS polyacrylamide gels and either stained with Coomassie blue (a) or blotted to nitrocellulose and reacted with Omni-probe (b). Proteins purified by affinity chromatography were electrophoresed in SDS polyacrylamide gels and either stained with Coomassie blue (c), or blotted to nitrocellulose and reacted with Omni-probe (d). The lanes contained: 1, p26-Δ36-His; 2, p26-Δ60-His; 3, p26-alpha-His; 4, p26-Δ40-His; 5, p26-Δ10-His; 6, p26-full-His; 7, vector lacking p26 cDNA. All lanes received 10 µl of sample. M, molecular mass markers of 97, 66, 45, 31, 21 and 14 kDa.

Fig. 3. **Oligomer formation by purified p26.** Bacterially produced p26 purified to apparent homogeneity by affinity chromatography was centrifuged at 200,000 x g for 21 h at 4 °C on 10-50% sucrose gradients. The gradients were fractionated and the A_{280} of each fraction was plotted against fraction number. The top of each gradient is to the right. The molecular mass markers, alpha-lactalbumin, 14.2 kDa; carbonic anhydrase, 29 kDa; bovine serum albumin, 66 kDa; alcohol dehydrogenase, 150 kDa; apoferritin, 443 kDa; and thyroglobulin, 669 kDa are indicated by numbered arrows.

Fig. 4. **p26 chaperone activity.** Bacterially produced p26 purified to apparent homogeneity was heated at 43 °C for 1 h with 150 nm citrate synthase and solution turbidity was measured at
A$_{360}$ p26 was at final concentrations of: a, 600 nM; b, 300 nM; c, 150 nM; d, 75 nM; e, 37.5 nM. The curves are: 1, no p26; 2, p26-alpha-His; 3, p26-NΔ60-His; 4, p26-NΔ36-His; 5, p26-CΔ40-His; 6, p26-CΔ10-His; 7, p26-full-His and they occupy the same relative positions in panels a-e. Panel f contains 150 nM citrate synthase incubated in the absence of other proteins (1) and with either 600 nM BSA (2) or 600 nM IgG (3).

**Fig. 5.** p26 synthesis in transfected COS-1 cells. Extracts prepared from COS-1 cells transfected with the pSecCMV eukaryotic expression vector containing p26 cDNAs were electrophoresed in SDS polyacrylamide gels and either stained with Coomassie blue (a) or transferred to nitrocellulose and stained with anti-p26 antibody followed by HRP-conjugated goat anti-rabbit IgG antibody (b). Each lane received 10 µl of extract from cells transfected with 1, p26-NΔ36; 2, p26-NΔ60; 3, p26-alpha; 4, p26-CΔ40; 5, p26-CΔ10; 6, p26-full. M, molecular mass markers of 97, 66, 45, 31, 21 and 14 kDa. Western blots containing extracts from COS-1 cells transfected with the pcDNA4/His.A expression vector containing p26 cDNAs and prepared as just described were stained with either anti-p26 antibody followed by HRP-conjugated goat anti-rabbit IgG antibody (c) or Omni-probe followed by HRP-conjugated goat anti-mouse IgG (d). Each lane in c and d received 10 µl of extract from cells transfected with 1, p26-full-His; 2, p26-NΔ60-His; p26-alpha-His.
Fig. 6. **p26 oligomer formation in transfected COS-1 cells.** Extracts prepared from COS-1 cells transiently transfected with p26-containing vectors were centrifuged in 10-50% sucrose gradients and 15 µl samples from each fraction were electrophoresed in 12.5% SDS polyacrylamide gels, transferred to nitrocellulose, and probed with either anti-p26 antibody followed by HRP-conjugated goat anti-rabbit IgG (a, c, d, e) or Omni-probe followed by HRP-conjugated goat anti-mouse IgG (b, f, g). The gradients contained, a, p26-full; b, p26-full-His; c, p26-CΔ10; d, p26-CΔ40; e, p26-NΔ36; f, p26-NΔ60-His; p26-alpha-His. The top of each gradient is to the right and fractions are numbered across the top. The positions of molecular mass markers, alpha-lactalbumin, 14.2; carbonic anhydrase, 29 kDa; bovine serum albumin, 66 kDa; alcohol dehydrogenase, 150 kDa; apoferritin, 443 kDa; and thyroglobulin, 669 kDa are indicated by numbered arrows.

Fig. 7. **p26 localization in transfected COS-1 cells.** COS-1 cells transfected with the p26 cDNA-containing vector pSecCMV were incubated with antibody to p26 followed by FITC-conjugated goat anti-rabbit IgG antibody (green) (a, c, d, e, f). Cells transfected with the p26 cDNA-containing vector pcDNA4/His.A were exposed to anti-p26 antibody followed by FITC-conjugated goat anti-rabbit IgG (green) (g, i) or to Omni-probe followed by FITC-conjugated goat anti-mouse IgG (green) (b, h, j). Nuclei were stained with propidium iodide (red) and samples were examined by confocal microscopy. a, p26-full; b, p26-full-His; c, p26-CΔ40; d, p26-CΔ10; e and f, p26-NΔ36; g, p26-NΔ60; h, p26-NΔ60-His; i, p26-alpha; j, p26-alpha-
His. The scale bar represents 50 \( \mu \)m and all figures are the same magnification.

**Fig. 8.** p26 oligomer size is unaffected by pH. Protein extracts obtained from *Artemia* cysts prepared at either pH 7.0 (a, c) or pH 6.5 (b, d), were centrifuged in 10-50\% continuous sucrose gradients either immediately after preparation (a, b), or following incubation at room temperature for 30 min (c, d). Fifteen \( \mu l \) fractions from each gradient were electrophoresed in 12.5\% SDS polyacrylamide gels, transferred to nitrocellulose, and probed with anti-p26 antibody followed by HRP-conjugated goat anti-rabbit IgG. The top of each gradient is to the right and fractions are numbered across the top. The positions of molecular mass markers, alpha-lactalbumin, 14.2; carbonic anhydrase, 29 kDa; bovine serum albumin, 66 kDa; alcohol dehydrogenase, 150 kDa; apoferritin, 443 kDa; and thyroglobulin, 669 kDa are indicated by numbered arrows.

**Fig. 9.** p26 oligomer size is unaffected by heat shock. Extracts prepared from heat shocked *Artemia* cysts and their nuclei were electrophoresed in SDS polyacrylamide gels and either stained with Coomassie blue (a) or blotted to nitrocellulose and stained with anti-p26 antibody (b). The lanes contained: 1, total cyst extract, heat shocked; 2, total cyst extract, control; 3, nuclear extract, heat shocked; 4, nuclear extract, control. Total extract from heated (c) and control (d) cysts and nuclear extracts from heated (e) and control (f) cysts were centrifuged in continuous 10-50\% sucrose gradients and 15 \( \mu l \) fractions from each gradient were electrophoresed in SDS polyacrylamide gels, transferred to nitrocellulose, and probed with anti-p26 antibody followed...
by HRP-conjugated goat anti-rabbit IgG. The top of each gradient is to the right and fractions are numbered across the top. The positions of molecular mass markers, alpha-lactalbumin, 14.2; carbonic anhydrase, 29 kDa; bovine serum albumin, 66 kDa; alcohol dehydrogenase, 150 kDa; apoferritin, 443 kDa; and thyroglobulin, 669 kDa are indicated by numbered arrows.

TABLE I

*Primers for site-directed mutagenesis of p26*

Truncated p26 variants were generated by site-directed mutagenesis using primers listed below in the 5’ to 3’ direction. The name of each p26 variant, either cDNA or protein, is indicated in the left-hand column. Full length p26, not shown in the table, is termed p26-full.

| p26 variant | Amino acid residues deleted | Primer sequence |
|-------------|----------------------------|-----------------|
| p26-NΔ36    | 1-36                       | CTCGTGCGGATCAAATGCCTTTCCGGAGAG<br>CTTCTCCGGAAGGGCATTTTGATCGGCACGAG |
| p26-NΔ60    | 1-60                       | CTCGTGCCGATCAAATGTCTTTGAGGACACAGC<br>GCTGTGTCCTCAAGGACATTTTGATCGGCACGAG |
| p26-CΔ40    | 153-192                    | GCTCAACACAGAAGCTTAAATCTGCATTC<br>GAATGCAGATTAACGTCTGTGTTGAGC |
| p26-CΔ10    | 183-192                    | GCTAGTTCAAAGCTACATAATCTGCATTC<br>GAATGCAGATTATGGAGTGGACTAGC |
| p26-alpha   | 1-60                       | CTCGTGCGGATCAAATGTCTTTGAGGACACAGC<br>GCTGTGTCCTCAAGGACATTTTGATCGGCACGAG<br>153-192 GCTCAACACAAGAAGCTTAAATCTGCATTC<br>GAATGCAGATTAACGTCTGTGTTGAGC |
TABLE II

Oligomerization of p26 produced in E. coli

The molecular mass of p26 oligomers produced by transformed E. coli was determined by reading the A280 of samples obtained by fractionation of sucrose density gradients. Monomer mass refers to the molecular mass of p26 polypeptides. Oligomer mass range represents the smallest to largest oligomers observed while oligomer mass peak refers to the mass of the most prevalent oligomer. Monomer number peak refers to the number of subunits in the most prevalent oligomer.

| P26 variant    | Monomer mass (kD) | Oligomer Mass range (kD) | Oligomer Mass peak (kD) | Monomer number peak |
|----------------|-------------------|--------------------------|------------------------|---------------------|
| p26-full-His   | 25.8              | 26-600                   | 150                    | 6                   |
| p26-NΔ36-His   | 22.0              | 22-110                   | 66                     | 3                   |
| p26-NΔ60-His   | 19.2              | 19-58                    | 19-38                  | 1-2                 |
| p26-alpha-His  | 15.5              | 16-62                    | 16                     | 1                   |
| p26-CΔ40-His   | 22.1              | 22-220                   | 66                     | 3                   |
| p26-CΔ10-His   | 24.9              | 25-500                   | 125                    | 5                   |
TABLE III

Oligomerization of p26 synthesized in transfected COS-1 cells

The molecular mass of p26 oligomers was determined by immunoprobing of samples electrophoresed in SDS polyacrylamide gels and transferred to western blots after sucrose gradient centrifugation. Monomer mass refers to the molecular mass of p26 polypeptides. Oligomer mass range represents the smallest to largest oligomers observed while oligomer mass peak refers to the mass of the most prevalent oligomer. Monomer number peak refers to the number of subunits in the most prevalent oligomer.

| p26 variant       | Monomer mass (kD) | Oligomer Mass range (kD) | Oligomer Mass peak (kD) | Monomer number (peak) |
|-------------------|-------------------|--------------------------|-------------------------|-----------------------|
| p26-full          | 20.8              | 21-437                   | 104                     | 5                     |
| p26-full-His      | 24.4              | 24-512                   | 146                     | 6                     |
| p26-CΔ10          | 19.9              | 20-438                   | 100                     | 5                     |
| p26-CΔ40          | 17.1              | 17-103                   | 51                      | 3                     |
| p26-NΔ36          | 17.0              | 17-68                    | 51                      | 3                     |
| p26-NΔ60-His      | 17.7              | 18-53                    | 18-35                   | 1-2                   |
| p26-alpha-His     | 14.0              | 14-56                    | 14                      | 1                     |
Figure 8
Figure 9
Oligomerization, chaperone activity and nuclear localization of p26, a small heat shock protein from artemia franciscana
Yu Sun, Marc Mansour, Julie A. Crack, Gillian L. Gass and Thomas H. MacRae

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