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Structural Organization of Procaryotic and Eucaryotic Hsp90

INFLUENCE OF DIVALENT CATIONS ON STRUCTURE AND FUNCTION*

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Ursula Jakob, Ines Meyer, Hans Bügl, Stefanie André, James C. A. Bardwell, and Johannes Buchner‡

From the Institut für Biophysik and Physikalische Biochemie, Universität Regensburg, 93040 Regensburg, Federal Republic of Germany

Hsp90 is a very abundant molecular chaperone that apparently helps to protect cellular proteins from denaturation upon temperature upshift. The unusual ability of Hsp90 to function under conditions where other proteins unfold prompted us to investigate the stability and structural organization of Hsp90 itself. Both procaryotic and eucaryotic members of the Hsp90 family were found to have very similar physicochemical properties: (i) they are stable against thermal unfolding up to at least 50 °C, (ii) they show biphasic, reversible unfolding transitions in guanidinium chloride, and (iii) their oligomerization state is strongly and rapidly affected by millimolar concentrations of divalent cations. In the presence of $MnCl_2$ and $MgCl_2$ defined changes in the quaternary structure of Hsp90 could be observed which resulted in a decrease in thermostability and an increased tendency to form larger aggregates. The addition of divalent cations also almost completely abolished the chaperone function of Hsp90 and induced release of folding intermediates of citrate synthase bound to Hsp90. These modulating effects of divalent cations on structure and function of Hsp90 *in vitro* represent a potential mechanism for regulation of Hsp90 chaperone action *in vivo*.

The exposure of organisms to elevated temperatures results in the overexpression of a specific subset of polypeptides, which have been termed heat shock proteins (Tissières *et al.*, 1974; Lindquist and Craig, 1988). They can be grouped in four classes, by molecular mass and homology: the small heat shock proteins (sHsps) with a molecular mass ranging from 15 to 32 kDa, the 70-kDa Hsps (Hsp70, DnaK), the 60-kDa Hsps (Hsp60, GroEL), and the 90-kDa proteins with a molecular mass ranging from 69 kDa in *Escherichia coli* (HtpG) to 90 kDa in humans (Hsp90) (Lindquist and Craig, 1988; Gething and Sambrook, 1992). All four classes are known to function as molecular chaperones and are thought to protect cells from the damaging effects of unphysiological high temperatures (for reviews, see Gething and Sambrook (1992), Jakob and Buchner (1994), Arrigo and Landry (1994), Parsell and Lindquist (1994), and Hendrick and Hartl (1993)).

Members of the Hsp90 family are the most abundant proteins in yeast under heat shock conditions, but also constitute up to 2% of the total cellular protein in yeast and various other cells during growth at normal temperatures (Lai *et al.*, 1984; Lindquist and Craig, 1988). While eucaryotic Hsp90s have been extensively studied in relation to their interaction with steroid receptors and kinases (Smith, 1993; Stancato *et al.*, 1993; Miyata and Yahara, 1992; Xu and Lindquist, 1993), only little is known about the function of Hsp90 under heat shock conditions and only a few studies have examined *E. coli* Hsp90 (Bardwell and Craig, 1987, 1988; Spence and Georgopoulos, 1989).

The Hsp90 family is one of the most highly conserved known; all members from bacteria to man share at least 40% amino acid identity with each other (Bardwell and Craig, 1987; Lindquist and Craig, 1988). The most predominant differences between *E. coli* and eucaryotic Hsp90s is (i) that *E. coli* Hsp90 is not essential, whereas yeast Hsp90 is essential; and (ii) *E. coli* Hsp90 lacks two charged domains (Bardwell and Craig, 1988; Borkovich *et al.*, 1989). Given the abundance and apparent importance of Hsp90 it is surprising how little is known about the structural organization and stability of this chaperone, compared with the extensive information that has been gleaned about other well conserved chaperones such as Hsp70 and GroEL (*cf.* Hendrick and Hartl (1993)). The stability of Hsp90 is of particular interest, since heat shock proteins are thought to function under conditions where other proteins unfold. We set out to determine and compare the molecular organization of eucaryotic and procaryotic Hsp90 using spectroscopic techniques and chemical cross-linking. We could demonstrate that all members of the Hsp90 family tested have similar physicochemical properties and that addition of divalent cations causes major changes in the quaternary structure which are accompanied by the instantaneous loss of function of Hsp90 as a molecular chaperone.

MATERIALS AND METHODS

Proteins—Hsp90 from bovine pancreas was purified as described (Wiech *et al.*, 1993). The protein concentration was determined according to Bradford (1976), using bovine serum albumin as a standard. Hsp90 from yeast and *E. coli* were purified as described below. Mitochondrial citrate synthase (EC 4.1.3.7.) was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). For citrate synthase the published extinction coefficient of 1.78 for a 1 mg/ml solution in a 1-cm cuvette was used (West *et al.*, 1990). Citrate synthase was stored in 50 mM Tris, 2 mM EDTA, pH 8.0. Concentrations of citrate synthase and Hsp90 are given for the dimeric form (Minami *et al.*, 1994). GroEL was the gift of Dr. Marion Schmidt (University of Regensburg, Germany). The monoclonal antibody against Hsp90 from yeast was from StressGen (Victoria, British Columbia, Canada). The polyclonal antiserum against *E. coli* Hsp90 was the kind gift of Dr. Michael Ehrmann (University of Konstanz, Germany).

Chemicals—The chemicals used for polyacrylamide gel electrophoresis and the hydroxyapatite resin were obtained from Bio-Rad. Diethylaminoethyl (DE52)-cellulose, S-Sepharose, and Superose 6 were from

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‡ To whom correspondence should be addressed: Institut für Biophysik and Physikalische Biochemie, Universität Regensburg, 93040 Regensburg, Germany. Tel.: 49-941-943-3039; Fax: 49-941-943-2813.

Pharmacia (Uppsala, Sweden). Guanidinium chloride (GdmCl)[1] ultrapure and HEPES were purchased from ICN (New York). Glutaraldehyde (GA, 25% (v/v) in H_20) and PMSF was obtained from Serva (Heidelberg, Germany). PABA, leupeptin, pepstatin, oxalacetic acid, acetyl-CoA, and a standard protein mix for size exclusion chromatography were purchased from Boehringer Mannheim GmbH. Nile red was obtained from Eastman Kodak. $MnCl_2$ and $(NH_4)_6Mo_7O_{24}$ were obtained from Sigma.

Buffers—Buffer A: 40 mM HEPES-KOH, 5 mM EDTA, 1 mM 1,4-dithioerythritol, 5% (v/v) glycerol, pH 7.5; buffer B: 20 mM sodium phosphate, pH 5.5; buffer C: 100 mM potassium phosphate, 5% (v/v) glycerol, pH 6.8; buffer D: 40 mM HEPES-KOH, 400 mM KCl, pH 7.5; buffer E: 40 mM HEPES-KOH, 20 mM KCl, 5% (v/v) glycerol, pH 7.5; buffer 1: 50 mM potassium phosphate, 20 mM KCl, 5% (v/v) glycerol, 1 mM EDTA, pH 7.5; buffer 2: 50 mM HEPES-KOH, 20 mM KCl, 5% (v/v) glycerol, 1 mM EDTA, pH 7.5; buffer 3: 20 mM potassium phosphate, 5% (v/v) glycerol, pH 6.8; buffer 4: 10 mM MES-HCl, 20 mM KCl, 5% (v/v) glycerol, pH 5.5.

Purification of Yeast Hsp90—Yeast Hsp90 was purified from a *hsc82* and *hsp82* deletion mutant strain ECUpep4 that carried *hsc82* on a 2μ URA3 overproducing plasmid (a kind gift of S. Lindquist). The yeast cells were grown at 25 °C in YPD medium (1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, 2% (w/v) D-(+)-glucose) to an A_{595} of 2.8, harvested, and washed twice in buffer A, containing a protease inhibitor mix (1 mM PMSF, 2.5 mM PABA, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). All following steps of the Hsp90 purification were carried out at 4 °C and the protease inhibitor mix was added during the first two steps of the purification. Cells were lysed with glass beads, adjusted to 0.1 M NaCl, centrifuged (48,000 × *g*, 60 min, 4 °C), and the supernatant was loaded onto a DE52 column equilibrated in buffer A containing 0.1 M NaCl. The column was washed with buffer A (+ 100 mM NaCl), and Hsp90 was eluted between 200 and 300 mM NaCl in a linear salt gradient. After centrifugation, the dialyzed (buffer B) Hsp90 pool was loaded onto S-Sepharose, equilibrated in buffer B. The major part of Hsp90 could be detected in the flow-through. The protein was concentrated (Amicon, YM30 membrane) and dialyzed against buffer C. It was then further purified on a hydroxyapatite column equilibrated in buffer C. Hsp90 eluted between 150–200 mM potassium phosphate. Concentrated fractions adjusted to 400 mM KCl were applied onto a Superose 6 column and eluted with buffer D. Hsp90 fractions were concentrated, dialyzed against buffer E, and stored at −70 °C. All purification steps were monitored by SDS-PAGE and immunoblots, using monoclonal antibodies against Hsp90. Yeast Hsp90 was about 98% pure as determined by densitometry.

Cloning and Purification of E. coli Hsp90—E. coli Hsp90 was expressed from its own heat shock promoter using the plasmid pBJ935. This plasmid contains the *E. coli* Hsp90 gene (*htpG*) cloned into the high copy number plasmid pUC19. It was constructed by cloning a 2.5-kilobase pair *Sal*I-*Eco*RI fragment containing the Hsp90 gene into the *Pst*I-*Eco*RI sites of pUC19. This fragment contains DNA extending from the *Sal*I site 165 base pairs upstream from the *E. coli* Hsp90 initiation codon to the *Eco*RI site 0.5 kilobase downstream from the termination codon. The *Sal*I site was first ligated to a *Pst*I site by cloning a kanamycin resistance cassette from pUC-4K into the *Sal*I site upstream from *E. coli* Hsp90 as described previously (Bardwell and Craig, 1987). This construct was introduced into the wild type *E. coli* strain MG1655 to generate the strain JCB 867. This strain grew normally at 30 °C and overproduced *E. coli* Hsp90 to >30% of the cellular protein. Expression at 37 °C or higher temperatures resulted in rapid plasmid loss. JCB 867 cells were grown in LB medium in the presence of 100 μg/ml ampicillin for 20 h at 30 °C to an A_{595} of 4.7. The cells were harvested by centrifugation and resuspended in buffer 1, containing 2 mM PMSF. All further purification steps were performed at 4 °C. *E. coli* Hsp90 was identified on SDS-PAGE and immunoblots with polyclonal antibodies against *E. coli* Hsp90 (provided by M. Ehrmann). The following purification protocol is based on the protocol originally used by Spence and Georgopoulos (1989). The cells were lysed by using French press at 18,000 p.s.i. for three cycles. The crude extract was centrifuged (48,000 × *g*, 30 min, 4 °C). Ammonium sulfate was added to a final concentration of 60% (w/v), and the lysate was incubated on ice for 30 min. The pellet of the ammonium sulfate precipitation was resuspended in buffer 2, containing 1 mM PMSF. After dialysis against buffer 2 and centrifugation, the supernatant was loaded onto a DE52-cellulose column, equilibrated in buffer 2. The proteins were eluted with a KCl gradient. *E. coli* Hsp90 eluted from the column between 150 and 300 mM KCl. The concentrated and dialyzed (buffer 3) pool was loaded onto a hydroxyapatite column equilibrated in buffer 3. *E. coli* Hsp90 eluted between 50 and 150 mM in a 20–200 mM potassium phosphate gradient. The pH of the protein solution was adjusted to 5.5 by dialysis against buffer 4. After centrifugation the supernatant was loaded onto a DE52 column, equilibrated in buffer 4. *E. coli* Hsp90 eluted at 150–250 mM KCl in buffer 4. Purified *E. coli* Hsp90 was nearly homogeneous as judged by SDS-PAGE (>98%). Finally, 80% (w/v) ammonium sulfate was added to the protein solution, and the ammonium sulfate precipitate was stored at 4 °C.

Determination of Protein Concentration—The protein concentration of yeast and *E. coli* Hsp90 was determined using the extinction coefficient of 0.72 and 1.25, respectively, given for a 1 mg/ml solution in a 1-cm cuvette. The extinction coefficients of the proteins were calculated according to Wetlaufer (1962) by using the aromatic amino acid composition of the proteins predicted from the DNA sequence. The determined protein concentrations could be confirmed using the tryptophan titration method for denatured proteins (Payot, 1975).

The UV spectra of the different Hsp90s show that the preparations are nucleotide-free indicated by a typical $A_{280/260}$ ratio of 1.75 for yeast Hsp90 and 1.83 for *E. coli* Hsp90.

Circular Dichroism Measurements—Near- and far-UV circular dichroism (CD) spectra were recorded in a Jasco J 500 A spectropolarimeter equipped with a Jasco DP 500 N data processor. Yeast and *E. coli* Hsp90 were dialyzed overnight against 40 mM HEPES-KOH, pH 7.5. Near-UV CD spectra were recorded from 250 to 350 nm in thermostatted 1-cm quartz cuvettes at 8 °C. The far-UV CD spectra were recorded from 200 to 250 nm at room temperature in 0.01-cm quartz cuvettes. All spectra were base line-corrected. To determine the influence of GdmCl on the secondary structure of Hsp90, the CD signal was recorded at 220 nm in 0.01-cm quartz cuvettes. The influence of ions on the far-UV CD spectrum of *E. coli* Hsp90 was monitored after incubating the samples in the presence of the respective ions for 30 min at 20 °C. Mean residue ellipticities for near- and far-UV CD spectra were calculated based on a mean residue molecular weight of 112.

Fluorescence and Light Scattering Measurements—The unfolding and refolding transitions of yeast and *E. coli* Hsp90 were monitored by measuring the change in intrinsic fluorescence. The measurements were performed in a Perkin-Elmer MPF44A luminescence spectrometer at 20 °C. The individual spectra were recorded from 290 to 350 nm in 1-cm quartz cells at an excitation wavelength of 285 nm. The spectral bandwidths were 5 and 10 nm for excitation and emission, respectively. To monitor the interaction of the dye nile red with accessible hydrophobic surfaces of the protein, fluorescence spectra were recorded from 560 to 700 nm at an excitation wavelength of 550 nm. The spectral bandwidths were 5 and 15 nm for excitation and emission, respectively. *E. coli* Hsp90 (0.1 mg/ml in 40 mM HEPES-KOH, pH 7.5) was incubated in the presence of 5 mM KCl, $MgCl_2$, $MnCl_2$, $(NH_4)_6Mo_7O_{24}$ or in the absence of ions for 30 min at room temperature. Nile red (0.25 mM in Me_2SO) was diluted 1:250 into the protein solutions and the samples were incubated for 5 min at room temperature before recording the spectra. To monitor the influence of $MgCl_2$ on the stability of bovine and yeast Hsp90, light scattering measurements were performed. Both excitation and emission wavelengths were set to 360 nm with 3 nm slit widths. Bovine and yeast Hsp90 (25 μg/ml) were incubated in various concentrations of $MgCl_2$ in 40 mM HEPES-KOH, pH 7.5, for 24 h at 20 °C.

GdmCl-induced Unfolding and Refolding of Yeast and E. coli Hsp90—GdmCl-induced unfolding of yeast and *E. coli* Hsp90 was performed by dilution of the proteins into various concentrations of GdmCl (40 mM HEPES-KOH, pH 7.5) ranging from 0 to 6 M. The respective protein concentrations are given in the figure legends. The samples were incubated for 24 h at 20 °C to achieve equilibrium. To study the influence of divalent ions on the stability of *E. coli* Hsp90, GdmCl-induced unfolding was performed as described in the presence and absence of 5 mM $MnCl_2$. For refolding experiments, yeast and *E. coli* Hsp90 was first denatured in 6 M GdmCl (40 mM HEPES-KOH, pH 7.5) for 6 h at 20 °C. Then the unfolded proteins were refolded by diluting into various concentrations of the denaturant ranging from 0 to 6 M GdmCl and further incubated for 24 h at 20 °C.

Temperature-induced Unfolding and Aggregation of Yeast and E. coli Hsp90—To monitor thermal unfolding and aggregation of yeast and *E. coli* Hsp90, fluorescence and light scattering measurements were performed in the temperature range from 30 °C to 75 °C. The measurements were carried out in stirred 1-cm quartz cells in a Perkin-Elmer

[1] The abbreviations used are: GdmCl, guanidinium chloride; GA, glutaraldehyde; PABA, 4-aminobenzamidine dihydrochloride; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride.

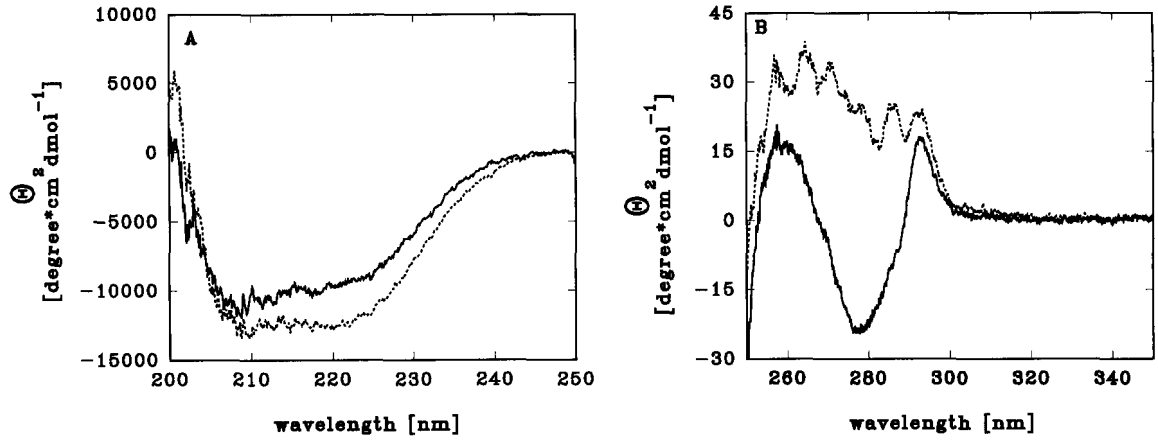

FIG. 1. **Secondary and tertiary structure of eucaryotic and procaryotic Hsp90.** *A*, far-UV CD spectra of 1 mg/ml yeast Hsp90 (—) and 1 mg/ml *E. coli* Hsp90 (· · · ·) in 40 mM HEPES-KOH, pH 7.5, at room temperature. *B*, near-UV CD spectra of 0.6 mg/ml yeast Hsp90 (—) and 0.8 mg/ml *E. coli* Hsp90 (· · · ·) in 40 mM HEPES-KOH, pH 7.5, at 8 °C.

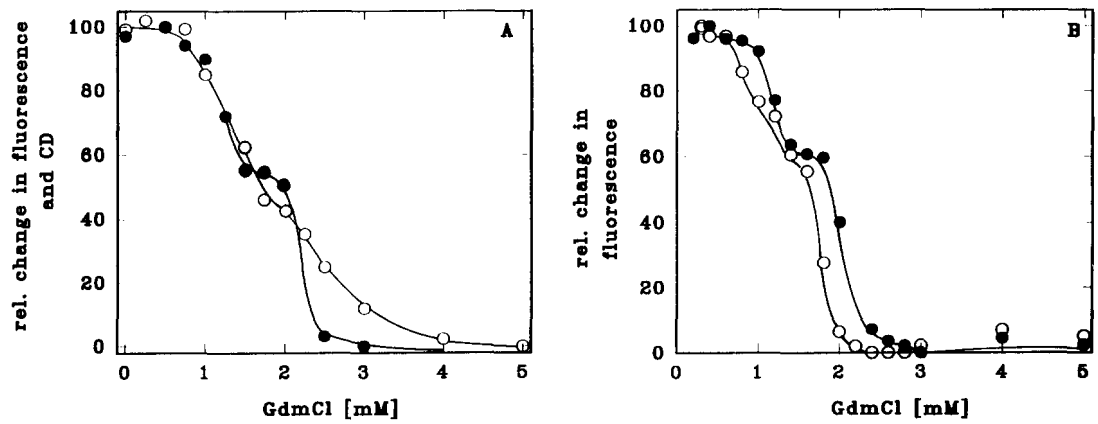

FIG. 2. **GdmCl-induced unfolding and refolding of eucaryotic and procaryotic Hsp90.** *A*, structural changes during the GdmCl-induced unfolding of *E. coli* Hsp90. *E. coli* Hsp90 (60 μg/ml in 40 mM HEPES-KOH, pH 7.5) was incubated in various concentrations of GdmCl for 24 h at 20 °C. The fluorescence signal at 325 nm was recorded (●). For monitoring changes in the secondary structure 1.4 mg/ml *E. coli* Hsp90 was incubated in various concentrations of GdmCl for 24 h at 20 °C before the CD signal at 220 nm was recorded (○). *B*, GdmCl-induced unfolding and refolding of yeast Hsp90. For unfolding experiments yeast Hsp90 (50 μg/ml in 40 mM HEPES-KOH, pH 7.5) was incubated in various concentrations of GdmCl for 24 h at 20 °C. To initiate refolding, denatured yeast Hsp90 (1 mg/ml in denatured in 6 M GdmCl) was diluted 1:20 into various concentrations of GdmCl and incubated for 24 h at 20 °C to achieve equilibrium. To monitor unfolding (●) and refolding (○) of yeast Hsp90, the fluorescence signal at 325 nm was recorded.

MPF44A luminescence spectrometer equipped with thermostatted cell holder connected to a thermoprogrammer. Yeast and *E. coli* Hsp90 were diluted into 40 mM HEPES-KOH, pH 7.5, at 25 °C (final concentration: 60 μg/ml). The tryptophan fluorescence of Hsp90 was measured at an excitation wavelength of 295 nm and an emission wavelength of 330 nm. The slit widths were set to 5 and 10 nm for emission and excitation, respectively. Light scattering measurements were performed in a stirred cuvette with both excitation and emission wavelength set to 360 nm, with 3 nm slit widths.

Gel Filtration of Yeast and E. coli Hsp90—For size exclusion chromatography, an analytical Superose 6 fast protein liquid chromatography column (25 ml) was used at a flow rate of 20 ml/h. The eluting protein was detected by fluorescence at 330 nm with excitation at 285 nm. The fluorescence detector (F 1000, Merck-Hitachi) was connected to an SP 4270 Integrator (Spectra-Physics) for evaluation of the peak areas. To determine the apparent molecular mass of yeast Hsp90 after 24 h incubation in the presence of various amounts of GdmCl, the elution buffer (40 mM HEPES-KOH, 20 mM KCl, pH 7.5) was supplemented with the respective GdmCl concentrations. Elution profiles of the standard protein mix (β-galactosidase, IgG, Fab, myoglobin) were also determined at the various concentrations of denaturants. With up to 1.5 M GdmCl present in the elution buffer, no significant change in the mobility of the standard proteins could be observed. To analyze the influence of $MnCl_2$ on the mobility of yeast and *E. coli* Hsp90, 5 mM $MnCl_2$ was added to the sample prior to loading it onto the gel filtration column. The elution buffer also contained 5 mM $MnCl_2$.

Cross-linking of Yeast and E. coli Hsp90—Chemical cross-linking of bovine, yeast, and *E. coli* Hsp90 was performed using GA as the cross-linking reagent. The optimal GA concentration was determined by titration and shown to be 20 mM. 4 μg of bovine Hsp90, yeast Hsp90, or *E. coli* Hsp90 were incubated in 20 μl of 40 mM HEPES-KOH, pH 7.5, for 30 min at room temperature in the presence or absence of various ions. The concentrations of these ions are given in the figure legends. For cross-linking, 1.6 μl 0.25 M GA (in 40 mM HEPES-KOH, pH 7.5) was added and the samples were incubated for 2 min at 37 °C. To stop the cross-linking process, the samples were supplemented with 5 μl of 1 M Tris-HCl, pH 8.0, and incubation was continued on ice. The cross-linked bands of Hsp90 were visualized by 3–20% gradient SDS-PAGE and quantified by densitometry.

Activity Assay for the Molecular Chaperone Function of Yeast and E. coli Hsp90s—To monitor the activity of bovine, yeast, and *E. coli* Hsp90 as molecular chaperones, their influence on the thermal unfolding and aggregation process of citrate synthase at 43 °C was monitored as described previously (Jakob *et al.*, 1995). The effects of ions on the activity of Hsp90 were studied by diluting the ions into the Hsp90 solution prior to the addition of citrate synthase.

RESULTS

Spectroscopic Properties of Yeast and E. coli Hsp90—To gain insight into the native structure of procaryotic and eucaryotic members of the Hsp90 family, fluorescence and circular dichroism spectra in the far- and near-UV region were recorded. The fluorescence spectra of yeast and *E. coli* Hsp90 corresponded

well to the known aromatic amino acid composition of the respective proteins (data not shown).

The far-UV CD spectra of yeast and *E. coli* Hsp90 showed an ellipticity maximum below 200 nm and two minima at 208 and 220 nm (Fig. 1*A*) in good agreement with data obtained for Hsp90 from higher eucaryotes (Csermely *et al.*, 1993). The secondary structure predicted for murine Hsp90 gives an average composition of 36% α-helix and 46% β-strands (Csermely *et al.*, 1993). Hsp90 from yeast and *E. coli* apparently have a similar secondary structure composition except the α-helical content of *E. coli* Hsp90 may be slightly higher.

Near-UV CD spectra of proteins represent a highly sensitive criterion for the native state of a protein and as such can be used as a "fingerprint" of the correctly folded conformation and tertiary structure (Schmid, 1989). Accordingly, significant differences in the near-UV spectra of yeast and *E. coli* Hsp90 could be detected (Fig. 1*B*). This spectroscopic characterization allowed us to investigate the stability of Hsp90 against chemical and thermal unfolding.

GdmCl-induced Folding Transitions of Yeast and E. coli Hsp90—Yeast and *E. coli* Hsp90 exhibited a similar biphasic unfolding behavior in the presence of increasing concentrations of the denaturant GdmCl as monitored by fluorescence (Fig. 2). The midpoint of the first transition occurred at about 1.2 M GdmCl, followed by a plateau ranging from 1.4 to 1.9 M GdmCl. The second transition with the midpoint at 2.2 M GdmCl resulted in a protein lacking detectable tertiary structure. To analyze whether the loss in tertiary structure is accompanied by or precedes the loss of secondary structure, we monitored GdmCl-induced changes in the CD signal at 220 nm (Fig. 2*A*), where the CD signal is dominated by secondary structural elements. Comparison of the changes observed by fluorescence and CD spectroscopy showed that in the first transition, some secondary and tertiary interactions are lost simultaneously (Fig. 2*A*). For the second transition, however, noncoincident transitions were obtained for the loss of secondary and tertiary structure. At 4.0 M GdmCl both yeast and *E. coli* Hsp90 were completely unfolded. To characterize the unfolding of yeast Hsp90 further, gel filtration chromatography was performed at different concentrations of GdmCl (Table I). Under native conditions, yeast Hsp90 eluted from the gel filtration column with a molecular mass of about 360 kDa, which corresponds to an apparent tetramer. High molecular weight complexes of Hsp90 eluting from size exclusion chromatography have been demonstrated previously for murine Hsp90 (Minami *et al.*, 1991) and for *E. coli* Hsp90 (Spence and Georgopoulos, 1989). However, the prevailing body of evidence suggests that Hsp90 is a dimer (Welch and Feramisco, 1982; Koyasu *et al.*, 1986; Radanyi *et al.*, 1989; Spence and Georgopoulos, 1989; Minami *et al.*, 1991). The addition of 10 mM GdmCl to sample and gel filtration buffer resulted in a mobility shift from the high molecular weight oligomers to the molecular mass of a dimeric protein (Table I). The formation of higher oligomeric species could be due to weak interactions between the subunits and does not influence the functional state of Hsp90. Dimeric yeast Hsp90 eluted from the gel filtration column at GdmCl concentrations ranging from 0.01 to 0.8 M. At 1.5 M GdmCl the mobility of yeast Hsp90 changed, presumably due to the partial unfolding and aggregation of the protein. To monitor the reversibility of Hsp90 unfolding, denatured Hsp90 was diluted into GdmCl concentrations ranging from 0 to 6 M. Besides a slight hysteresis between the unfolding and refolding curves of yeast Hsp90, the transitions obtained were very similar, showing that the unfolding of yeast and *E. coli* Hsp90 is reversible (Fig. 2*B* and data not shown). Slight aggregation occurring during the refolding of both Hsp90s decreased the yield of renatured protein. After removal of the aggregates far-UV CD spectra and gel filtration analysis of the refolded yeast Hsp90 revealed a fully renatured, native-like protein (data not shown).

TABLE I

Size exclusion chromatography of yeast Hsp90 in the presence of various concentrations of GdmCl

5 μg of yeast Hsp90 were incubated in various concentrations of GdmCl for 24 h at 20 °C and were loaded onto a Superose 6 column (25 ml) which was equilibrated with the respective concentrations of GdmCl in 40 mM HEPES-KOH, 20 mM KCl. The flow rate was 20 ml/h.

GdmCl	Retention time	Apparent molecular mass
M	*min*	*kDa*
0	42.0	360
0.01	45.0	200
0.80	45.0	200
1.50	32.7	>800

Thermal Unfolding of Yeast and E. coli Hsp90—Since eucaryotic and procaryotic Hsp90s are able to protect proteins from thermal inactivation and aggregation (Jakob *et al.*, 1995), it was interesting to investigate the thermostability of these heat shock proteins. We monitored tryptophan fluorescence as well as the light scattering signal of yeast and *E. coli* Hsp90 in the temperature range from 30 to 75 °C (Fig. 3). Up to 50 °C, the tryptophan fluorescence signal of yeast Hsp90 decreased linearly with increasing temperature (Fig. 3*A*), corresponding to the decrease in specific fluorescence of tryptophan with in-

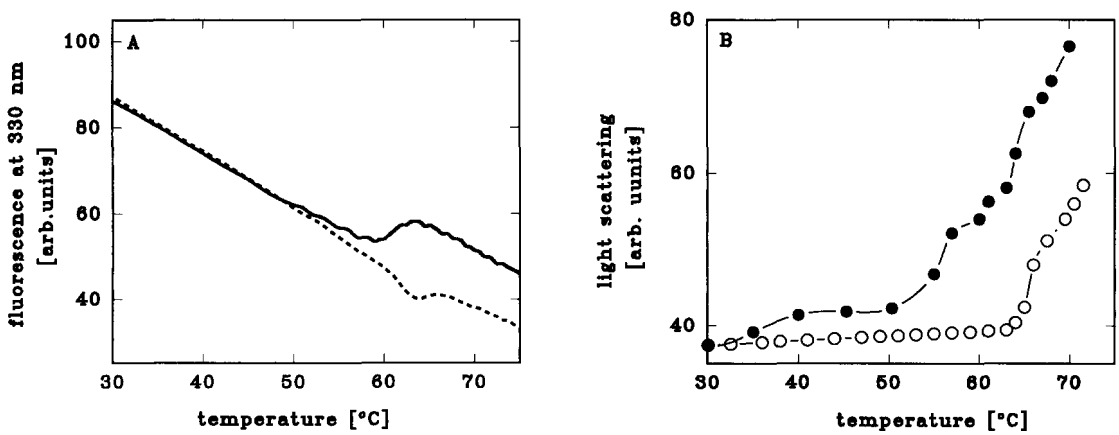

FIG. 3. **Thermal unfolding and aggregation of procaryotic and eucaryotic Hsp90.** Yeast Hsp90 and *E. coli* Hsp90 were diluted into incubation buffer (40 mM HEPES-KOH, pH 7.5) at 25 °C. The final protein concentration was 60 μg/ml. The heating rate was 1 °C/min. *A*, thermal unfolding transition of yeast (–) and *E. coli* Hsp90 (· · · ·). The fluorescence signal at 330 nm was monitored. *B*, thermal aggregation of yeast (●) and *E. coli* Hsp90 (○). To monitor aggregation the change in light scattering signal was followed.

creasing temperature (Schmid, 1989). Between 59 and 64 °C a sharp increase in the intrinsic tryptophan fluorescence could be observed with the midpoint of the transition at 61 °C. This is likely to reflect the thermal unfolding of the protein. Whether unfolding results in an increase or decrease of fluorescence is most likely determined by changes in the micro-environment of aromatic residues and can thus not be interpreted further. Due to the increasing influence of aggregation, the subsequent decrease in fluorescence at higher temperatures occurred at a rate greater than the linear decline of free tryptophan fluorescence at increasing temperature. Qualitatively similar results were obtained with *E. coli* Hsp90 (Fig. 3). The thermal unfolding transition of the procaryotic member of the Hsp90 family occurred at about 64 °C (Fig. 3*A*) and was also accompanied by aggregation (Fig. 3*B*). Hsp90 from *E. coli* and yeast are thus more thermostable than Hsp90 from higher eucaryotes where the thermal transition of Hsp90 occurred at 50 °C (Lanks *et al.*, 1992).

Influence of Divalent Cations on the Cross-linking Pattern of Hsp90—To analyze the quaternary structure of Hsp90 under native conditions, we performed cross-linking studies with GA. In the presence of 2 mM EDTA, only 20% Hsp90 could be cross-linked as dimers at the given Hsp90 and optimized GA concentration (Fig. 4). No higher cross-linked species could be detected on the gradient gel. This is in contrast to the results, obtained by size exclusion chromatography under native-like conditions, but in agreement with the analysis of eucaryotic Hsp90 where mainly dimeric species were detected (Welch and Feramisco, 1982; Koyasu *et al.*, 1986; Radanyi *et al.*, 1989; Minami *et al.*, 1991). This confirms the hypothesis that the interactions between the subunits responsible for the appearance of high molecular weight complexes in gel filtration experiments are rather weak. Because it has been speculated that Hsp90 may bind ATP (Csermely and Kahn, 1991), comparative cross-linking experiments were performed with nucleotide-free Hsp90 (see "Materials and Methods") in the presence of 5 mM MgATP, 5 mM ATP, and 5 mM $MgCl_2$ (data not shown). While ATP and MgATP did not show any influence on the cross-linking pattern, $MgCl_2$ shifted the equilibrium between monomeric and dimeric Hsp90 significantly toward higher cross-linked species, including tetramers (Fig. 4, *A* and *C*). Therefore we examined the effect of various monovalent and divalent ions as well as transition state metals on the association state of bovine, yeast, and *E. coli* Hsp90. Fig. 4*A* shows the cross-linking pattern of yeast Hsp90 in the presence of various amounts of KCl, $MnCl_2$, $MgCl_2$, and $(NH_4)_6Mo_7O_{24}$. Although monovalent ions and the transition state metal molybdate had no effect on the cross-linking pattern of Hsp90, the presence of divalent cations increased the amount of Hsp90 cross-linked into dimers and tetramers. $MgCl_2$ (Fig. 4, *A* and *C*) and $MnCl_2$ (Fig. 4, *A* and *B*) had an equivalent effect. As shown in Fig. 4*B*, the amount of monomeric species decreased with raising the $MnCl_2$ concentration, whereas the amount of cross-linked dimers and tetramers increased. Higher $MnCl_2$ concentrations resulted in an increase in the amount of tetrameric cross-linked Hsp90 at the expense of dimers. The change in quaternary structure could also be confirmed by size exclusion chromatography of *E. coli* Hsp90. Under native-like conditions (40 mM HEPES-KOH, 20 mM KCl) the protein eluted from a Superose 6 column as a 540-kDa oligomer, corresponding to an apparent octamer. No monomeric or dimeric forms were detected. The presence of 5 mM $MnCl_2$ in the sample and the elution buffer induced a significant change in the elution profile of *E. coli* Hsp90. The mobility corresponded now to an apparent tetrameric protein (approximately 240 kDa). The effects of divalent cations on the quaternary structure of Hsp90 from higher

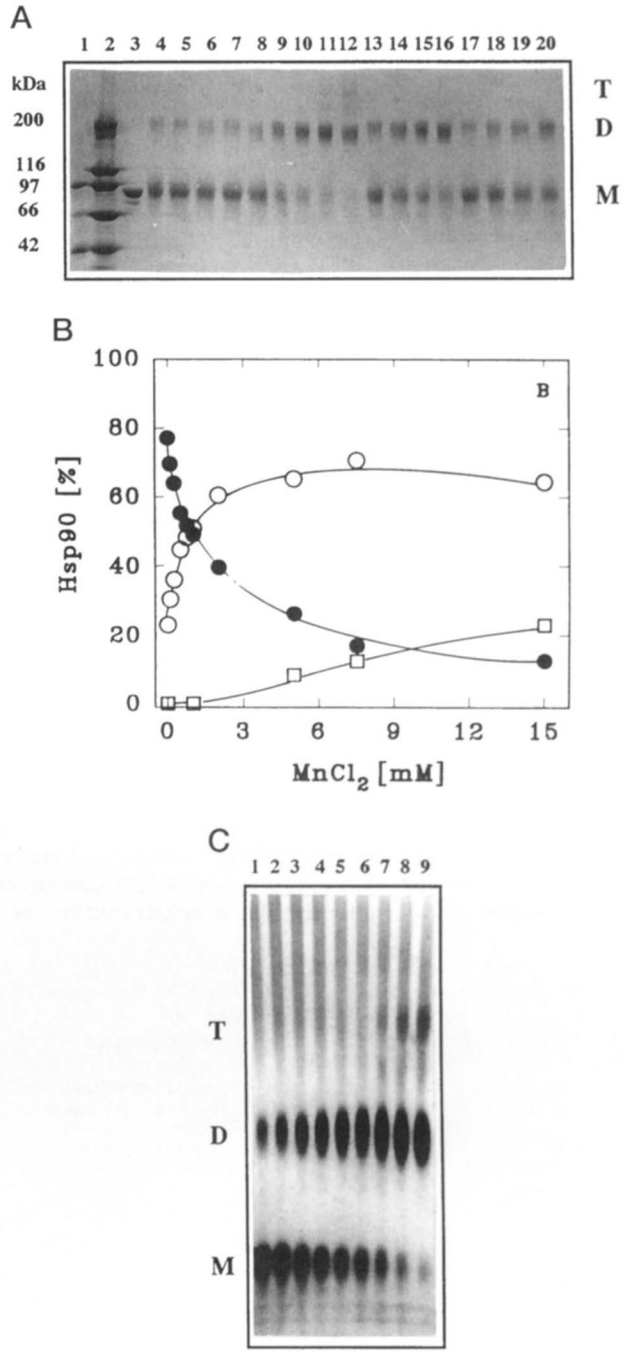

FIG. 4. **Cross-linking of eucaryotic and procaryotic Hsp90 with GA.** *A*, cross-linking of yeast Hsp90 in the presence of various concentrations of mono- and divalent cations and the transition state metal molybdate. Yeast Hsp90 was cross-linked with GA after 30-min incubation in the presence of 2 mM EDTA (*lane 4*) and increasing concentrations (1, 2, 5, and 10 mM) KCl (*lanes 5–8*), $MnCl_2$ (*lanes 9–12*), $MgCl_2$ (*lanes 13–16*), and $(NH_4)_6Mo_7O_{24}$ (*lanes 17–20*). Similar results were obtained with bovine and *E. coli* Hsp90. *Lanes 1, 2,* and *3* represent low and high molecular weight standards and noncross-linked yeast Hsp90, respectively. *M, D,* and *T* represent monomeric, dimeric, and tetrameric cross-linked species. *B*, $MnCl_2$-induced oligomerization of *E. coli* Hsp90. *E. coli* Hsp90 was incubated at room temperature in the presence of increasing amounts of $MnCl_2$ before cross-linking with GA was performed. The formation of monomeric (●), dimeric (○), and tetrameric (□) cross-linked species was analyzed by densitometry of the respective bands after SDS-PAGE. *C*, cross-linking pattern of bovine Hsp90 in the presence of $MgCl_2$. Bovine Hsp90 was incubated at room temperature in the presence of various amounts of $MgCl_2$ before cross-linking with GA was performed. The incubation reaction contained 2 mM EDTA (*lane 1*) or increasing concentrations of $MgCl_2$. *Lanes 2* to *13* refer to 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, and 10.0 mM $MgCl_2$ in the incubation reaction. *M, D,* and *T* represent monomeric, dimeric, and tetrameric cross-linked species.

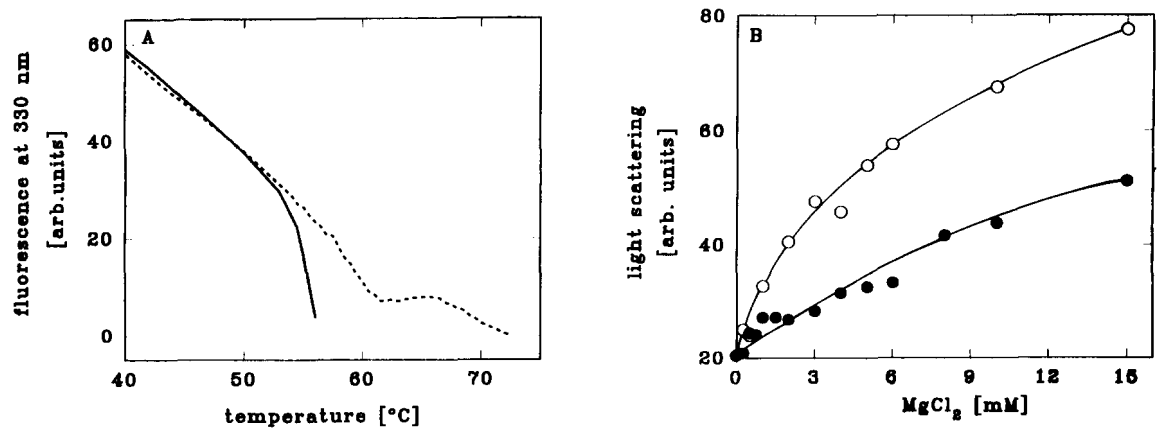

FIG. 5. ***Influence of divalent cations on the stability of Hsp90.*** *A*, thermal unfolding of *E. coli* Hsp90 in the absence and presence of divalent cations. The changes in tertiary structure of *E. coli* Hsp90 (60 μg/ml in 40 mM HEPES-KOH, pH 7.5) in the absence (· · · ·) or presence (—) of 5 mM $MnCl_2$ were monitored by fluorescence at 330 nm. The heating rate was 0.4 °C. *B*, $MgCl_2$ induced aggregation of yeast and bovine Hsp90. Light scattering measurements were performed after incubation of 25 μg/ml yeast Hsp90 (○) and 25 μg/ml bovine Hsp90 (●) in the presence of various concentrations of $MgCl_2$ for 24 h at 20 °C.

eucaryotes were similar to their effects on *E. coli* Hsp90 (Fig. 4*C*). Thus, we conclude that divalent cation-induced conformational changes represent a general property of members of the Hsp90 family. Interestingly, the change in Hsp90 conformation seemed to be a rapid process occurring within 1 min after addition of divalent cations (data not shown).

Effects of Divalent Cations on Structure and Stability of Yeast and E. coli Hsp90—To examine, whether incubation in the presence of divalent cations induces conformational changes in the secondary and tertiary structure of Hsp90, fluorescence, and CD measurements were performed. No changes in the secondary structure and tertiary structure of yeast and *E. coli* Hsp90 could be detected after a 30-min incubation in the presence of 5 mM $MgCl_2$ and $MnCl_2$ (data not shown). Additional fluorescence measurements with the hydrophobic dye nile red did not reveal major changes in the accessible hydrophobic surfaces of the proteins (data not shown).

Comparison of the GdmCl- and thermal-induced transition curves of Hsp90 in the absence and presence of divalent cations should provide further insight in their influence on structure and stability of Hsp90. Although the GdmCl-induced transition of *E. coli* Hsp90 in the presence of $MnCl_2$ was comparable with the transition in the absence of additional components (Fig. 2*A*), significant differences in the thermal stability of *E. coli* Hsp90 in the presence of $MnCl_2$ could be detected (Fig. 5*A*). The midpoint temperature of the thermal unfolding transition of Hsp90 in the presence of divalent cations was far below the temperature, where unfolding took place in the absence of ions. The cation-induced tendency of Hsp90 to form larger aggregates could be confirmed by measuring the light scattering of Hsp90, which was incubated for 24 h in the presence of various amounts of $MgCl_2$, ranging from 0 to 15 mM (Fig. 5*B*). This aggregation process did not result in a major change in fluorescence, explaining the similar fluorescence spectra in the absence and presence of divalent cations (data not shown).

Influence of Divalent Cations on the Function of Hsp90 as Molecular Chaperones in Vitro—We examined the influence of Hsp90 on the thermal aggregation and inactivation of citrate synthase at 43 °C. In the absence of divalent cations, *E. coli* Hsp90 was able to suppress the thermal aggregation of citrate synthase significantly (Fig. 6*A*) and to stabilize the protein by interacting with early unfolding intermediates (Jakob *et al.*, 1995). However, in the presence of 1 mM $MgCl_2$ the ability of *E. coli* Hsp90 to function as a molecular chaperone was almost completely abolished (Fig. 6*A*). Maximum aggregation was reached within 20 min both in the complete absence of Hsp90 or in the presence of Hsp90 and cations. Only a slight delay in the increase of the light scattering signal of citrate synthase in the presence of *E. coli* Hsp90 and cations could be observed initially. The aggregation process of citrate synthase itself was not affected by the presence of $MgCl_2$ (Fig. 6*A*). Experiments with bovine Hsp90 gave similar results (data not shown).

Confirming the results we obtained with light scattering measurements, we also observed that Hsp90 had much less effect on the stabilization of citrate synthase against thermal inactivation in the presence of divalent cations than in their absence (Fig. 6*B*). Again, the presence of $MgCl_2$ or $MnCl_2$ in the incubation reaction had no influence on the inactivation process of citrate synthase in the absence of Hsp90 or in the presence of bovine serum albumin (data not shown). To examine whether divalent cations exert also an influence on preformed Hsp90·citrate synthase complexes, $MgCl_2$ was added 5 min after start of the inactivation process (Fig. 6*C*). This resulted in a sudden increase in the light scattering signal, indicating that citrate synthase was immediately released from Hsp90 and aggregated. Hsp90 itself did not reveal any detectable aggregation in the presence of divalent cations in the time range of the experiment (data not shown). To test whether GroEL is able to recognize released citrate synthase molecules in the presence of divalent cations, 5 s after the addition of $MgCl_2$ GroEL was added. As shown in Fig. 6*C*, substoichiometric amounts of GroEL were capable of binding these intermediates and forming a stable complex, thus suppressing aggregation. Citrate synthase was still a substrate protein for GroEL in the presence of divalent cations and made it thus less likely that divalent cations induce conformational changes in citrate synthase itself, preventing its interaction with chaperones.

DISCUSSION

Two general conclusions can be drawn from our studies: 1) eucaryotic and procaryotic members of the Hsp90 family are very similar in their physicochemical properties, although they are derived from widely divergent organisms. These similarities mirror the high degree of conservation in the proteins concerning sequence, structure, and function. 2) The oligomeric state of Hsp90 is strongly affected by the addition of millimolar quantities of divalent cations and is accompanied by the simultaneous and rapid loss of activity. These association forms are aggregation-sensitive and probably represent inactive precursors of higher aggregates of Hsp90.

Yeast and *E. coli* Hsp90 have similar secondary and quaternary structure as measured by various physicochemical tech-

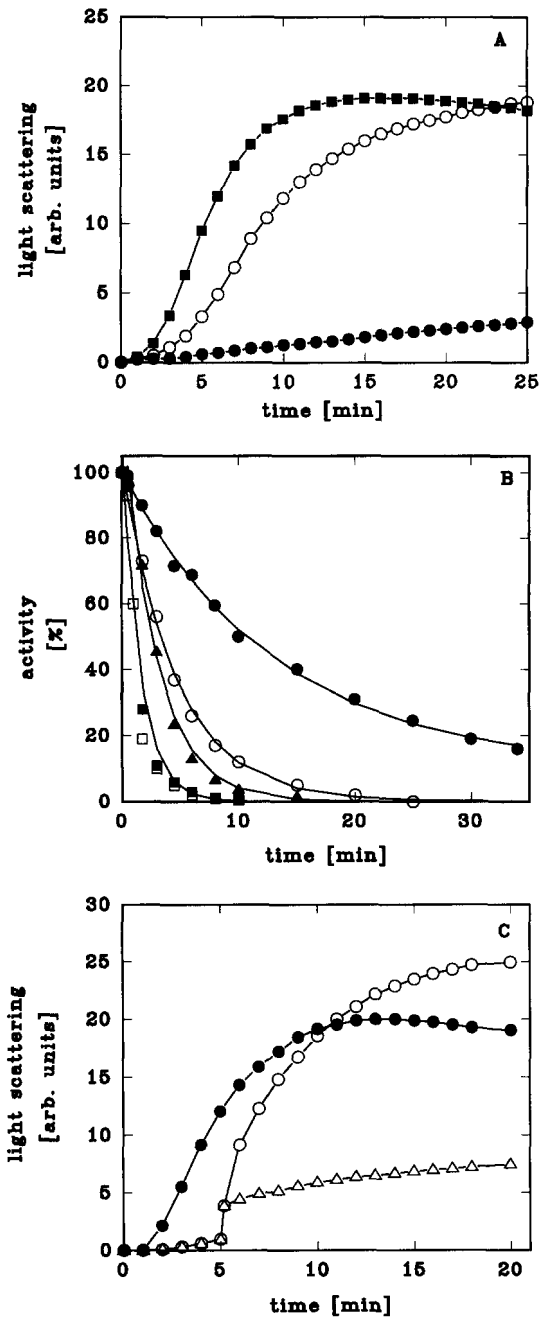

FIG. 6. **Divalent cation-induced change in the chaperone activity of Hsp90 *in vitro*.** *A*, influence of $MgCl_2$ on the *E. coli* Hsp90-induced suppression of thermal aggregation of citrate synthase at 43 °C. 0.075 μM citrate synthase was incubated in the presence of 0.6 μM *E. coli* Hsp90 with (○) or without (●) 1 mM $MgCl_2$ in the incubation reaction. Similar results were obtained with yeast and bovine Hsp90. *Closed squares* represent the light scattering signal of citrate synthase ± 1 mM $MgCl_2$ in the absence of 0.6 μM *E. coli* Hsp90. *B*, influence of $MnCl_2$ on the yeast Hsp90-induced stabilization of thermally inactivating citrate synthase at 43 °C. Citrate synthase (0.075 μM) was incubated with 0.6 μM yeast Hsp90 in the absence of divalent cations (●) or in the presence of (○) 1 mM $MnCl_2$ of (▲) 3 mM $MnCl_2$. Control experiments of citrate synthase without Hsp90 were also performed in the presence of 3 mM $MnCl_2$ (■) or in the absence of $MnCl_2$ (□). Qualitatively similar data were obtained with *E. coli* and bovine Hsp90. *C*, $MgCl_2$-induced release of citrate synthase intermediates from preformed Hsp90 · citrate synthase complexes. Citrate synthase (0.075 μM) was incubated in the absence (●) or presence of 0.15 μM bovine Hsp90 at 43 °C. After 5 min of incubation in the presence of Hsp90, $MgCl_2$ (5 mM) was added followed 5 s later by the addition of (○) 0.3 μM bovine serum albumin or (△) 0.1 μM GroEL (14-mer). The aggregation kinetics were monitored by light scattering.

niques. Size exclusion chromatography of Hsp90 under native-like conditions confirmed earlier experiments that suggested eucaryotic and procaryotic proteins may elute as higher oligomeric species (Minami *et al.*, 1991; Spence and Georgopoulos, 1989). Since addition of small amounts of GdmCl leads to the dissociation of Hsp90 into dimers, it seems likely that the formation of higher oligomers of Hsp90 is due to weak interactions between the individual dimers. This is in agreement with earlier studies suggesting the dimer to be the native state of association (Welch and Feramisco, 1982; Koyasu *et al.*, 1986; Radanyi *et al.*, 1989; Minami *et al.*, 1991). Since Hsp90 is fully active under conditions under which it elutes as a dimeric protein as well as under conditions where the elution profile reveals an oligomeric protein (Wiech *et al.*, 1992; Jakob *et al.*, 1995), in contrast to other Hsps such as GroEL and GroES, oligomerization is clearly not essential for function.

Heat shock proteins are known to function under conditions under which other proteins unfold. It was therefore not surprising to find that yeast and *E. coli* Hsp90 unfold at temperatures that are more than 15 °C above the maximal growth temperature of the organisms. GdmCl-induced unfolding of yeast and *E. coli* Hsp90, which was monitored by following changes in the tertiary structure, exerted a biphasic transition curve with a plateau ranging from 1.4 to 1.9 M GdmCl, suggesting the formation of some unfolding intermediate. In contrast to thermal denaturation, the GdmCl-induced unfolding of yeast and *E. coli* Hsp90 seemed to be reversible processes, as monitored by fluorescence, CD, and gel filtration.

The observation that the cross-linking pattern of both proteins changed significantly in the presence of divalent cations prompted us to investigate the influence of ions on the structural organization and stability of Hsp90. Only 20% of the proteins could be cross-linked as dimeric proteins. The appearance of higher oligomeric species was dependent on the concentration of divalent cations in the incubation reaction. At the concentration used, GA is in the monomeric form and can covalently link arginine and lysine residues which are about 8 Å apart (Kawahara *et al.*, 1992; Peters and Richards, 1977). The different cross-linking pattern of Hsp90 as a result of the incubation with divalent cations can therefore be interpreted in two ways: (i) the divalent cation-induced conformational changes result in different oligomeric states or (ii) the cations change the conformation of the subunits of the oligomer so that two residues are brought in the appropriate distance for successful cross-linking. A similar influence of divalent ions on the cross-linking pattern of GroEL was recently observed (Azem *et al.*, 1994). Divalent cations affect the structure of GroEL subunits at contact sites and therefore increase the velocity of intra- and intermolecular (between the GroEL-7 mers) cross-linking with GA. These divalent cation-induced changes in the conformation of GroEL are distinct from the influence of $MgCl_2$ on the ATPase activity of GroEL (Azem *et al.*, 1994) and do not affect its function. In contrast to the stabilizing effects of divalent cations on urea-induced unfolding of GroEL, no significant stabilization by cations of yeast and *E. coli* Hsp90 against chemical denaturation was observed. Instead, incubation of Hsp90 for 24 h in the presence of various concentrations of divalent cations led to an increasing tendency of aggregation. Furthermore, the thermal unfolding transition of Hsp90 occurred at lower temperatures in the presence of $MnCl_2$. These results and gel filtration experiments in the presence of divalent cations led to the conclusion that divalent cation-induced changes in the conformation of Hsp90 produce different oligomeric states, which could represent precursors of larger aggregates.

An *in vivo* relevance of divalent cations on the function of Hsp90 is suggested by the finding that the functional activity of

Hsp90 as a molecular chaperone *in vitro* is dramatically reduced in the presence of divalent cations. Yeast and *E. coli* Hsp90 are normally able to decelerate the thermal inactivation of citrate synthase by binding to early unfolding intermediates of the enzyme thus reducing the amount of aggregation-sensitive intermediates (Jakob *et al.*, 1995). In the presence of divalent cations, this function of Hsp90 was almost completely inhibited. This was true for yeast and *E. coli* Hsp90 as well as for Hsp90 from higher eucaryotes. Addition of $MgCl_2$ to a complex formed between Hsp90 and citrate synthase resulted in a sudden increase in the light scattering signal. This indicated that the cation-induced conformational changes in Hsp90 occur very quickly and induce release of unfolding intermediates of citrate synthase from Hsp90.

The cation-induced release of folding intermediates from Hsp90 suggests an *in vivo* regulatory role of these ions in the function of the chaperone. It remains to be seen if this effect of cations on the function of members of the Hsp90 family extends to other chaperones such as Hsp70 and the small Hsps.

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