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Characterization of Purified Avian 90,000-Da Heat Shock Protein

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The so-called 90,000-Da heat shock protein (hsp90) from chicken liver has been purified and physically characterized in the presence of high levels of the serine phosphatase inhibitor fluoride. The protein is an elongated dimer with a molecular weight of 160,000 and a frictional ratio of 1.6. On two-dimensional electrophoresis it exhibits several isoelectric forms lying between pH 5.1 and 5.8. It contains an average of 5.8 mol of covalently bound phosphate per dimer and is thus extensively phosphorylated. Analysis of the ultraviolet spectrum showed the purified protein to be free of nucleotide-containing components. Molybdate has been shown to stabilize complexes between the 90,000-Da heat shock protein and steroid receptors. However, molybdate has no effect on the sedimentation of the purified heat shock protein. Proteins structurally related to hsp90 have been reported to penetrate the endoplasmic reticulum. However, when purified hsp90 was tested using the partition method of Bordier, which distinguishes hydrophilic and lipophilic proteins, it partitioned totally into the aqueous phase. © 1988 Academic Press, Inc.

When cells are stressed by high temperature, they increase the synthesis of a small number of proteins called heat shock proteins (1, 2). These proteins are also produced in response to a number of other cytotoxic agents such as transition metals, amino acid analogs and sulfhydryl reagents (3, 4). These heat shock proteins, or stress proteins, appear to protect the cells producing them (5). The 90,000-Da heat shock protein (hsp90)[2] is one of three major eukaryotic heat shock proteins (6). It is also a major ubiquitous protein in unstressed cells, comprising approximately 1% of cytosolic protein (7).

Hsp90 and an unidentified 50,000-Da phosphoprotein have been shown to form a complex with the Rous sarcoma virus transforming tyrosine kinase (8). The kinase is a plasma membrane protein, and it has been suggested that hsp90 is involved in the transport of the kinase to the membrane (9). Hsp90 and the 50,000-Da protein form similar complexes with the tyrosine kinases from two other avian retroviruses (10). Hsp90 has also been shown to be a component of the 8 S forms of steroid receptors (11, 12). In addition, hsp90 has recently been shown to bind to actin filaments (13).

Hsp90 was extensively purified from HeLa cells by Welch and Feramisco (6). Their purification did not, however, separate hsp90 from the 100,000-Da heat shock protein. By adding a heparin-agarose column to the procedures used by Welch and Feramisco we have been able to purify the hsp90 from chicken liver to apparent homogeneity. Purification to homogeneity enabled us to answer questions about the physical structure of hsp90 which could not be validly addressed with less pure preparations. Since hsp90 contains phosphoserine, our purification buffers contained the serine phosphatase inhibitor fluoride (14, 15).

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[2] Abbreviations used: hsp90, 90,000-Da heat shock protein; SDS, sodium dodecyl sulfate.

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METHODS

Materials. Ampholines pH 3-10 and pH 5-8 were from Pharmacia. Isoelectric focusing standards, ovalbumin (grade II crystallized), and bovine serum albumin (98-99%, remainder mostly globulins) were obtained from Sigma Chemical Co.

Egg white (spray dried, phosphate analyzed) was supplied by the NBCo Biochemicals Division, ICN Biomedicals Inc.

Purification of hsp90. Our purification was based on the method used by Welch and Feramisco to purify hsp90 from HeLa cells (6). We modified their procedure principally by including additional protease and serine phosphatase inhibitors throughout the purification. The livers of female White Leghorn chickens were homogenized in 4 vol of 20 mM Tris, 20 mM NaCl, 50 mM NaF, 4 mM EDTA, 15 mM mercaptoethanol, pH 7.6. The homogenization buffer (Buffer A) included 0.25 mM phenylmethylsulfonyl fluoride and 2 μg/ml of pepstatin A and leupeptin. The two peptide protease inhibitors were included in all subsequent buffers. The homogenate was centrifuged for 60 min at 105,000*g*. Lipid was removed by aspiration and the high-speed supernatant was run onto a DE-52 column. The column was washed with buffer A and eluted with a gradient running from Buffer A to Buffer A containing 500 mM NaCl. Hsp90 was located by slab gel electrophoresis and dialyzed into 20 mM potassium phosphate, 4 mM EDTA, 50 mM NaF, 15 mM mercaptoethanol (Buffer B). It was next run onto a hydroxylapatite column, washed with Buffer B and eluted with a gradient running to Buffer B containing 300 mM potassium phosphate. The hydroxylapatite peak was dialyzed and concentrated into Buffer A using a Schleicher & Scheull dialyzer/concentrator and chromatographed on Sephacryl S-300 in Buffer A. Welch and Feramisco reported that their S-300 hsp90 peak contained one major contaminant, the 100,000-Da heat shock protein. We also saw an S-300 peak with a detectable contaminant running at 100,000 Da. We were able to separate hsp90 from the contaminant by adding an additional step to the purification, chromatography on heparin-agarose. The S-300 peak was dialyzed and concentrated into 20 mM Tris, pH 7.6, 20 mM NaCl, 25 mM NaF, 0.5 mM EDTA, 15 mM mercaptoethanol (Buffer C). It was then run onto a 2 ml heparin-agarose column, washed with 20 ml of Buffer C, and eluted with a 30-ml gradient running from Buffer C to Buffer C containing 500 mM NaCl. On this column the hsp90 eluted at 150 mM NaCl and the 100,000-Da contaminant and some other minor contaminants eluted at higher salt levels. Preparations starting with 100 g of liver yielded approximately 5 mg of purified hsp90. The purified protein was dialyzed into buffer A and stored in the refrigerator or diluted 1:1 with glycerol and stored at −20°C. The latter procedure was preferable since storage at −20°C greatly slowed the degradation of hsp90 by a contaminating protease.

Two-dimensional electrophoresis. Two-dimensional electrophoresis was performed by the method of O'Farrell *et al.* (16). For the first dimension (1.5 mm tube gels) 4% Ampholines pH 5-8 and 1.0% Ampholines pH 3-10 were utilized at 10,000 V-h. In the second dimension electrophoresis was performed on 14% slab gels (17). Four micrograms of each standard and 30 μg of sample were loaded per run. The sample was first precipitated with 20% trichloroacetic acid followed by two 1:1 ethanol:ether washes of the pelleted material.

Determining protein phosphate content. The phosphate assay was performed according to Ames (18) with the following sample preparation: 200 μg of bovine serum albumin was added as a carrier for the precipitation of sample in 1 ml of 20% trichloroacetic acid. The pelleted material was washed twice with 1 ml ice-cold ethanol:ether (1:1). Egg white protein containing a known phosphate content was taken through this procedure as a control.

Protein was determined by the method of Schaffner and Weissmann (19) with bovine serum albumin as standard.

Gel filtration. Samples were chromatographed on a 51 × 1.6-cm column of Sephacryl S-300, and 1.4-ml fractions were collected. The buffer contained 20 mM Tris, pH 7.6, 100 mM NaCl, 25 mM NaF, 0.1 mM EDTA, and 15 mM mercaptoethanol. Blue dextran was used to estimate the void volume and glucose (determined by the anthrone method) was used to estimate the total included volume. The column was standardized with ovalbumin (30.5 Å Stokes radius), bovine serum albumin (35.5), apoferritin (61.0), and thyroglobulin (85.0). The Stokes radii of hsp90 preparations were estimated using a linear correlation of $K_D^{1/3}$ with the Stokes radii of the standards (20).

Glycerol gradients. Linear (10-35%) glycerol gradients were prepared by the layering diffusion method of Stone (21) in buffer containing 20 mM Tris, pH 7.6, 100 mM NaCl, 25 mM NaF, 0.1 mM EDTA, and 15 mM mercaptoethanol. Samples (0.2 ml) were layered on the gradients and tubes were centrifuged at 40,000 rpm and 4°C in an SW 50.1 rotor for 16 h. Fractions were collected using an ISCO Model 640 density gradient fractionator. External standards of aldolase (7.9 S) and [^{14}C]ovalbumin (3.7 S) were used to estimate sedimentation coefficients. Aldolase and hsp90 were located using the Bradford (22) protein assay. For experiments investigating the effects of molybdate, gradients were run in 50 mM potassium phosphate, 50 mM potassium chloride, 10 mM thioglycerol, pH 7.0 (12).

Removal of hsp90 by antibody. The monoclonal antibody used, 7Δ11, was prepared against the 90,000-Da protein from chicken oviduct which forms an 8 S complex with progesterone receptor (23). This pro-

tein has recently been shown to be hsp90 (24). Purified hsp90 (45 μg) was mixed with 100 μg of antibody in 200 μl of 5 mM Tris, 80 mM NaCl, pH 7.0. The buffer also contained 66 μg/ml of bovine serum albumin as carrier. Controls were run by adding buffer lacking antibody. Samples were incubated on ice for 2 h. Meanwhile 0.8-ml agitated batches of Sigma anti-mouse IgG agarose were washed into the Tris buffer. After the slurry was added to microfuge tubes containing the samples, the tubes were capped and shaken for 3 h in the cold room. The tubes were then centrifuged, and aliquots of supernatant were precipitated and analyzed by slab gel electrophoresis.

Partition into Triton X-114. The method of Bordier was followed (25).

RESULTS AND DISCUSSION

By adding a heparin-agarose column to the method of Welch and Feramisco (6), we were able to purify the hsp90 from chicken liver to a form appearing homogeneous on a slab gel stained with Coomassie blue (Fig. 1A). To confirm that the protein we had purified was hsp90 we used the monoclonal antibody 7Δ11. This antibody had been prepared in the laboratory of Dr. David Toft against the hsp90 of chicken oviduct. The antibody complexed with our protein and caused it to bind when anti-mouse IgG-agarose was added. Spinning down the agarose removed the protein. For this experiment bovine serum albumin was added to the protein solution as a carrier and a control. The bovine serum albumin was unaffected by the antibody (Fig. 1B, lanes 1, 2).

The purified hsp90 was analyzed by two-dimensional electrophoresis. Isoelectric focusing standards were run at the same time to check the effectiveness of the focusing, and to permit estimation of hsp90 p*I* values. While standards on either side of it appeared as discrete spots, the hsp90 focused as a long band (Fig. 2). A similar pattern was shown by the HeLa hsp90 (26). Since the pH gradient was not linear across the slab gel, we could conclude only that all forms of hsp90 had p*I* values more basic than pH 5.1 and more acidic than 5.8.

Proteins which exist in multiple phosphorylated forms can show patterns like that shown by hsp90 on two-dimensional electrophoresis. Hsp90 has been found to

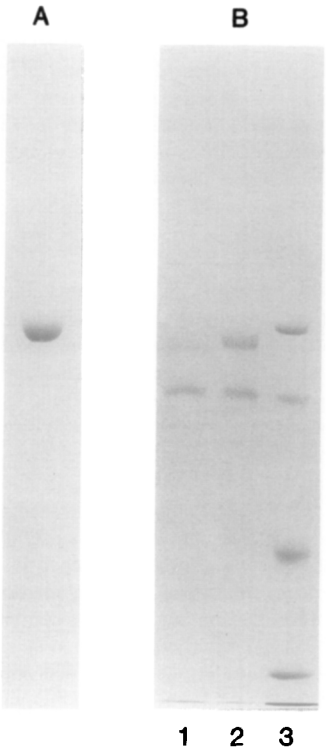

FIG. 1. Analysis of purified hsp90 by slab gel electrophoresis. (A) Hsp90 from the heparin-agarose column (17 μg) was run on an 8% slab gel and stained with Coomassie blue. (B) Removal of purified hsp90 by monoclonal antibody. The hsp90 peak from the heparin-agarose column was incubated in buffer containing bovine serum albumin and containing (1) or lacking (2) the monoclonal antibody 7Δ11. The samples were then incubated with anti-mouse IgG agarose and centrifuged to remove the agarose. Supernatant aliquots were precipitated with trichloroacetic acid, washed with 1:1 ethanol:ether and subjected to electrophoresis. Lane 3 contains the following standards: phosphorylase *b*, 92,500 Da; bovine serum albumin, 66,000; ovalbumin, 45,000; and carbonic anhydrase, 31,000.

be a phosphoprotein in all tissues which have been examined (26). Both the hsp90 bound to the Rous sarcoma virus transforming protein and the hsp90 bound to steroid receptors contain phosphoserine (27, 28). Accordingly we decided to analyze the phosphate content of the purified hsp90. The buffers used during purification included high levels of the phosphatase inhibitor fluoride in an attempt to minimize dephosphorylation during the purification procedure (15).

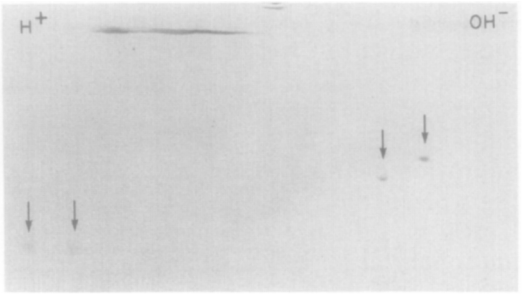

FIG. 2. Two-dimensional gel analysis of purified hsp90. Arrows indicate the positions of standards with known p*I* values. From left to right the standards and their p*I* values are: soybean trypsin inhibitor, 4.55; β-lactoglobulin A, 5.13; bovine carbonic anhydrase B, 5.85; human carbonic anhydrase B, 6.57.

Aliquots of purified hsp90 were precipitated and washed in the presence of carrier bovine serum albumin. They were washed twice in the presence of magnesium nitrate, and the released phosphate was estimated colorimetrically by the method of Ames (18) as an acidic complex with molybdate. The estimated phosphate content of hsp90 was 2.9 ± 0.1 mol/mol monomer. The data represented a total of five determinations, two on one hsp90 batch and three on a batch purified separately. For this calculation an estimated molecular weight of 82,000 was used for the hsp90 monomer. The hsp90 proteins from three species have been completely sequenced. The human version has a molecular weight of approximately 83,000 (29). Versions from *Drosophila melanogaster* and yeast have molecular weights of approximately 82,000 and 81,000, respectively (30, 31).

Glycogen phosphorylase is a relatively abundant protein with a molecular weight near that of hsp90. Since the apparent molecular weights of proteins on SDS gels can vary with changes in phosphorylation state, we decided to check on the possible identity of these two proteins. Aliquots of hsp90 and of phosphorylase *b* from rabbit muscle were run on 8% slab gels. The bands were cut out, equilibrated and digested with papain using the method of Cleveland *et al.* (32). On electrophoresis of the digestion products, the two proteins showed distinctly different patterns, indicating a lack of identity (data not shown).

The ultraviolet spectrum of the purified hsp90 was examined (Fig. 3) to see whether the protein copurified with any nucleotide-containing material. The spectrum observed showed a peak at 279 nm and a shoulder at 285 nm corresponding to the tyrosine spectrum. Minor shoulders at 260 and 269 nm were also seen, probably corresponding to the absorption spectrum phenylalanine (33). The OD_{280}/OD_{260} ratio of hsp90 was 1.72. Samples of bovine serum albumin and ovalbumin examined in the same way had ratios of 1.68 and 1.82, respectively. Thus the ultraviolet absorbance spectrum gave no evidence suggesting that the purified hsp90 contained attached nucleotides.

We also investigated the size and shape of the avian hsp90. Samples of hsp90 purified through the heparin-agarose step were analyzed by gel filtration on a Sephacryl S-300 column (Fig. 4a) and by glycerol gradient centrifugation in the same buffer (Fig. 4b). Analysis of four samples by gel filtration gave an estimated Stokes radius of 64 ± 2 Å. Analysis of three samples by centrifugation gave an estimated sedimentation coefficient of 5.8 ± 0.1 S. Using these values after the method of Siegel and Monty (20) we calculated a molecular weight for the protein of

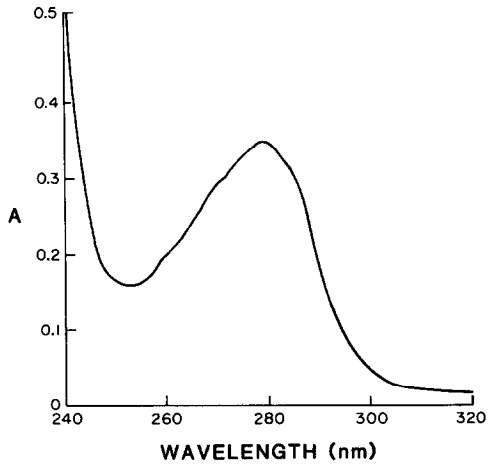

FIG. 3. Ultraviolet-absorption spectrum of hsp90. Protein concentration was 0.5 mg/ml in 10 mM Tris, pH 7.6, 50 mM KCl.

160,000 ± 10,000, and a frictional ratio of 1.6. For these calculations a partial specific volume of 0.732 cm^3/g was used (34).

These data indicate that the purified hsp90 exists as a dimer with a rather high frictional ratio. The frictional ratio can be used to estimate an axial ratio for the protein, treating it as a prolate ellipsoid (35). For this calculation the water of hydration was assumed to be 0.2 g/g protein (34), and the calculated axial ratio was 9. Koyasu *et al.* (13) have recently reported similar values for the size and shape of hsp90 from a mouse lymphoma cell line. Steroid receptors, to which hsp90 binds, have high frictional and axial ratios also, as does the complex which hsp90 forms with steroid receptors (36). Renoir *et al.* (37) have proposed that the 8 S form of progesterone receptor is composed of one copy of the receptor itself and two copies of hsp90.

The estimated molecular weight of 160,000 ± 10,000 for the hsp90 dimer is consistent with the size of the monomers in the three species for which the hsp90 sequences have been determined. These have molecular weights ranging from 81,000 to 83,000 (29-31). The molecular weight of the hsp90 monomer was originally estimated to be 90,000 based on the migration of this protein on a slab gel in the presence of sodium dodecyl sulfate. Hsp90 is multiply phosphorylated, and phosphorylation has been shown to cause overestimation of protein molecular weights in this type of system (38).

Kelley and Schlesinger (14) reported that the heat shock protein from chicken embryo fibroblasts eluted from a gel filtration column with an apparent molecular weight of 510,000. In our studies the chicken liver hsp90 eluted slightly faster than apoferritin (M_r 443,000), thus behaving like a globular protein with a molecular weight near 500,000. It seems quite possible that the form of hsp90 detected by Kelley and Schlesinger was an elongated dimer like the one we describe.

In the presence of molybdate, the 8 S complexes formed between hsp90 and steroid receptors can be purified to apparent

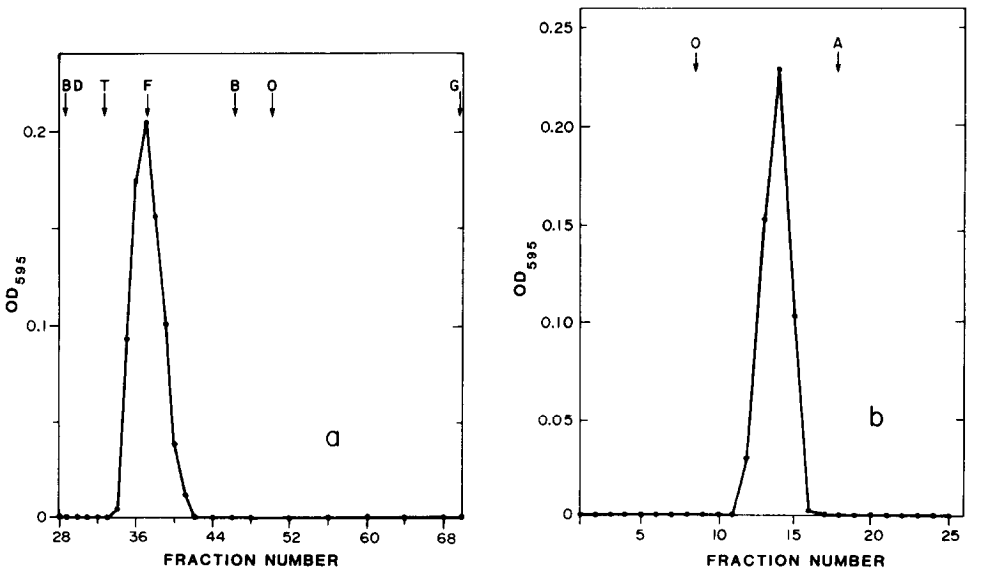

FIG. 4. Analysis of purified hsp90 by gel filtration (a) and by glycerol gradient sedimentation (b). (a) Hsp90 from the heparin-agarose column was chromatographed on a Sephacryl S-300 column. Arrows indicate the elution volumes of blue dextran (BD), thyroglobulin (T), apoferritin (F), bovine serum albumin (B), ovalbumin (O), and glucose (G). (b) Sedimentation of hsp90 through a 10-35% glycerol gradient. Arrows indicate the positions of the external standards aldolase (A, 7.9 S) and ovalbumin (O, 3.7 S). Hsp90 was located using the Bradford protein assay (22).

homogeneity. On removal of molybdate the complexes come apart, and the components sediment at lower S values (39). We decided to check whether the presence of molybdate had any detectable effect on the sedimentation behavior of purified hsp90. The hsp90 was dialyzed into a pH 7.0 phosphate buffer like one previously used to purify an 8 S complex between hsp90 and progesterone receptor (12). Its sedimentation was found to be unaffected by the presence or absence of 10 mM molybdate. Results were as follows: +molybdate, 6.3 ± 0.2 S (n = 6); −molybdate, 6.2 ± 0.2 S (n = 6).

There have been several reports recently indicating that proteins related to hsp90 are found associated with membranes. Ullrich *et al.* reported that a mouse tumor-specific transplantation antigen was found both in the cytosol and on the plasma membrane (40). This antigen showed sequence homology to the yeast and *Drosophila* versions of hsp90, and may be a variant of the murine hsp90. ERp99, an abundant murine transmembrane glycoprotein of the endoplasmic reticulum has also been reported to show extensive homology with the *Drosophila* version of hsp90 (41). Similarly, the glucose-regulated protein grp94, a membrane protein of the endoplasmic reticulum, was reported to have extensive homology with the yeast and *Drosophila* hsp90 versions (42). Erp99 and grp94 share identical amino terminal sequences and may be the same protein (41).

In view of these results, we decided to test whether the purified hsp90 showed any tendency to partition like a membrane protein. Bordier (25) has shown that hydrophilic proteins and integral membrane proteins can be distinguished on the basis of how they partition between an aqueous phase and a detergent phase of Triton X-114. Purified hsp90 was partitioned using his method and examined by gel electrophoresis (Fig. 5). The protein showed no detectable tendency to partition into the detergent phase.

Hsp90 is a multiply phosphorylated dimer which binds to steroid receptors, several oncogene kinases, and actin. Phosphorylation has been shown to regulate the affinities with which proteins bind each other (43, 44). It will be interesting to learn whether the phosphorylation state of hsp90 affects its affinities for the proteins which it binds.

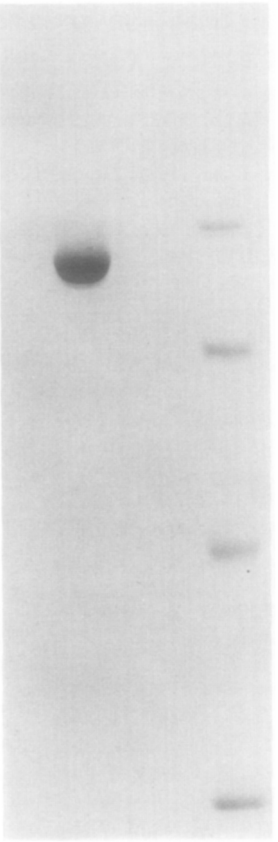

FIG. 5. Partition of hsp90 between aqueous phase and Triton X-114. Hsp90 was dialyzed into a homogeneous solution containing Triton X-114 at 0°C. On heating at 30°C, the Triton X-114 separated to form a detergent phase. The two phases were washed according to the method of Bordier (25) and examined by gel electrophoresis. Lane 1, aqueous phase; lane 2, detergent phase.

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