Cooperative Action of Hsp70, Hsp90, and DnaJ Proteins in Protein Renaturation†

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ABSTRACT: The proteins required for the repair of damaged proteins in the eukaryotic cytoplasm remain largely uncharacterized. The renaturation of thermally denatured firefly luciferase readily occurs in rabbit reticulocyte lysate by an ATP-dependent process. Earlier studies had shown that this chaperoning activity could be reconstituted, in part, using purified preparations of hsp70 and hsp90. We have extended the description of this system by clarifying the importance of hsp70 and hsp90 and have tested for additional factors that enhance renaturation. Using mutant hsp70 proteins, we have shown that hsp70 is required for luciferase renaturation. We have also found that hsp70 and hsp90 preparations purified by common procedures were contaminated with low levels of DnaJ proteins that are essential for the renaturing activity. When hsp70 and hsp90 preparations free of DnaJ proteins are used, the system must be supplemented with a DnaJ protein to obtain renaturation activity. The yeast DnaJ protein, YDJ-1, was found to be very effective for this purpose. Although significant renaturation can occur with only hsp70 and DnaJ proteins, hsp90 also contributes to the renaturation process, both in the complex environment of reticulocyte lysate and in a purified system. However, using highly purified hsp90 and geldanamycin, a specific inhibitor of hsp90 function, we have determined that hsp90 is not an essential component of the renaturation system. The contribution of hsp90 to renaturation is only partially blocked by geldanamycin, suggesting that this protein may influence activity in more than one way. This study indicates that hsp70, hsp90, and DnaJ proteins function cooperatively to renature damaged proteins in the eukaryotic cytoplasm and provides a framework by which additional components can be identified and individual chaperone contributions can be investigated.

Molecular chaperones play important roles in a wide variety of cellular processes. Members of the hsp70[1] family of molecular chaperones are involved in the synthesis and folding of nascent polypeptides, protein translocation, assembly of protein complexes, antigen presentation, protection from thermal stress, protein degradation, and uncoating of clathrin-coated vesicles (Hendrick & Hartl, 1993; Georgopoulos & Welch, 1993). Although hsp70 may act alone in some of these cellular roles, it often requires the cooperation of co-chaperones, such as DnaJ and GrpE in bacteria, or DnaJ homologs in eukaryotes. In some instances, this cooperativity among chaperones may extend to the hsp60 family of chaperones, thus forming a chaperone pathway along which substrates are passed from one group of chaperones to the next (Cheng et al., 1989; Manning-Krieg et al., 1991; Langer et al., 1992).

One of the most studied and best characterized systems for hsp70 function is in bacteria where DnaK (bacterial hsp70), DnaJ, and GrpE cooperate in several biological processes. In *Escherichia coli* these three proteins interact in bacteriophage lambda and P1 replication (Zylicz et al., 1989; Alfano & McMacken, 1989; Wickner et al., 1991; Ang et al., 1991) and in the renaturation of thermally damaged proteins (Schröder et al., 1993). These proteins are also sufficient to refold chemically denatured proteins *in vitro*, although some proteins apparently also require GroEL and GroES for complete refolding (Langer et al., 1992; Szabo et al., 1994; Buchberger et al., 1994). The requirements for the renaturation of damaged proteins in the eukaryotic cytoplasm are less clear. Evidence is accumulating which suggests that hsp70, hsp90, and hsp104 are involved in the reactivation and resolubilization of thermally damaged proteins in eukaryotes (Parsell et al., 1994; Schumacher et al., 1994; Ziemienowicz et al., 1995; Jacob et al., 1995; Yonehara et al., 1996). However, GrpE homologs have not been identified in the eukaryotic cytoplasm, and the role of eukaryotic DnaJ proteins in protein renaturation has not been fully investigated.

Several laboratories have studied protein folding and processing activities in rabbit reticulocyte lysate (Schumacher

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[1] Abbreviations: hsp, heat shock protein; GA, geldanamycin; DTT, dithiothreitol; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TRiC, TCP-1 ring complex; TB, Tris buffer; AB, assay buffer; YDJ, yeast homolog of DnaJ; HDJ, human homolog of DnaJ; P90, hsp90 purified as in text; Hhsp90, human hsp90.

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et al., 1994; Frydman et al., 1994; Kruse et al., 1995; Hansen et al., 1994; Nimmesgern & Hartl, 1993; Smith et al., 1990, 1992; Scherrer et al., 1990; Matts et al., 1992; Murakami et al., 1988; Chirico et al., 1988; Pelham, 1988; Mattingly et al., 1993). This system is rich in components needed for protein synthesis and folding, including molecular chaperones such as hsp70, hsp90, and TRiC, and it appears to retain some of the complexities of the eukaryotic cytoplasm. Unlike most studies which have addressed the folding of nacent polypeptide chains or chemically denatured proteins, we have developed an assay to investigate the chaperoning activity needed to correct the folding of mildly denatured (partially folded) proteins. We have used thermally denatured firefly luciferase to characterize the renaturation activity in reticulocyte lysate (Schumacher et al., 1994). Renaturation in this case is very efficient and requires ATP and potassium, but it is not dependent on the TRiC proteins that have been demonstrated to chaperone protein folding in some other systems (Frydman et al., 1992; Yaffe et al., 1992; Gao et al., 1992). Additionally, samples containing both hsp70 and hsp90 were able to support limited ATP-dependent luciferase renaturation. In most cases, neither protein sample had significant renaturation activity alone, suggesting that these two proteins cooperated in protein renaturation in reticulocyte lysate. However, renaturation by purified heat shock proteins is much less efficient than renaturation by whole reticulocyte lysate. We, therefore, set out to identify other factors that were required for more efficient renaturation of thermally denatured proteins in eukaryotes. We had noticed variation among preparations of hsp70 and hsp90 regarding their contributions to renaturing activity. Thus, these preparations were analyzed for the presence of factors that contribute to their activity. A second objective was to confirm the roles of hsp70 and hsp90 in the protein renaturation system.

Our analysis of protein fractions containing stimulating activity for renaturation revealed the essential role of DnaJ proteins in the process. The requirement of hsp70 was confirmed; however, while hsp90 could enhance the renaturation process, it was not an essential component.

EXPERIMENTAL PROCEDURES

Buffers. Tris buffer (TB): 10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 50 mM KCl, and 2 mM dithiothreitol (DTT). Assay buffer (AB): 25 mM Tricine-HCl, pH 7.8, 8 mM $MgSO_4$, 0.1 mM EDTA, 12 mM DTT, 100 μM D-luciferin, 240 μM coenzyme A, and 0.5 mM ATP. Stability buffer (SB): 25 mM Tricine-HCl, pH 7.8, 8 mM $MgSO_4$, 0.1 mM EDTA, 10 mg/mL BSA, 10% glycerol, and 0.25% Triton X-100.

Luciferase Renaturation Assay. Firefly luciferase (Sigma), in SB at 100 nM, was denatured to less than 1% of the original luciferase activity by incubation for ~15 min at 40 °C. Denatured luciferase was incubated on ice for 10 min and then diluted 10-fold into untreated rabbit reticulocyte lysate (Green Hector, Oregon, WI) or purified protein samples and analyzed for renaturing activity at 25 °C. Renaturation samples were prepared in TB with 2 mM ATP and an ATP-regenerating system [10 mM phosphocreatine (Sigma) and 3.5 units of creatine kinase (type I, rabbit muscle, Sigma) per 100 μL of sample]. To assay for luciferase activity, 5 μL of renaturing sample was added to 120 μL of AB, and light production was measured for 15 s in a Turner luminometer. In all experiments, the activity was expressed as a percent of control samples which were handled identically but without denaturation at 40 °C.

Purification of Chicken Hsp90 (P90). Hsp90 was purified from chicken liver essentially as described previously (Welch & Feramisco, 1982; Iannotti et al., 1988) omitting the gel filtration step. Briefly, chicken liver cytosol was prepared in 20 mM Tris-HCl, pH 7.5, 20 mM KCl, 20 mM NaF, 5 mM β-mercaptoethanol, and 4 mM EDTA plus the protease inhibitors, phenylmethanesulfonyl fluoride (0.25 mM), pepstatin (2 μg/mL), and leupeptin (2 μg/mL). This was fractionated on DEAE-cellulose which was initially washed with 0.15 M KCl followed by KCl gradient elution (0.15−0.5 M). Fractions containing hsp90 were identified by gel electrophoresis, pooled, dialyzed in 20 mM potassium phosphate, pH 7.5, 1 mM EDTA, 20 mM NaF, and 5 mM β-mercaptoethanol, and applied to a hydroxylapatite column. Proteins were eluted with a 10−500 mM phosphate gradient. Hsp90-containing fractions identified by gel electrophoresis were pooled, concentrated by ammonium sulfate precipitation (75% saturation), dialyzed in 10 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM EDTA, and 10% glycerol, and stored at −70 °C. Samples were dialyzed into TB before being used in renaturation assays.

Purification of Human Hsp90 (Hhsp90). A baculovirus system for the overexpression of human hsp90β in SF9 cells was used and has been described previously (Alnemri & Litwack, 1993). The harvested cells were suspended in 4 volumes of buffer and disrupted by sonication. Hsp90 was purified from the cytosol fraction as described above for chicken hsp90. The yield of hsp90 was about 1 mg/100 mL culture, and the final purity was >90%.

Purification of Hsp70. The DEAE-cellulose fraction eluted with 0.15 M KCl (see chicken hsp90 purification above) was used as a source of hsp70. This was fractionated on a column of ATP−agarose (Sigma A-2767) as described by Schlossman et al. (1984). Pooled fractions of hsp70 were concentrated by precipitation with ammonium sulfate (75% saturation), and the precipitate was dissolved and dialyzed into TB and stored at −70 °C. In some cases, hsp70 was further purified to remove DnaJ proteins. Hsp70 purified as above was applied to a 16/60 Superdex 200 sizing column (Pharmacia) equilibrated with TB. The hsp70 fractions eluting in the monomeric range were pooled and concentrated using Centriplus-10 and Centricon-10 units (Amicon). Based on Western analysis, hsp70 purified in this manner was free of DnaJ proteins.

Bacterial Expression and Purification of YDJ-1. A bacterial expression system for YDJ-1p was generously supplied by Dr. Avrom Caplan and has been described previously (Caplan et al., 1992). Bacterial pellets were suspended and sonicated in 3 volumes of 10 mM Tris, pH 7.5, 1 mM EDTA, and 5 mM DTT plus 1 mM protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, Calbiochem). After centrifugation (100000*g*, 1 h), the extract was loaded on a DEAE-cellulose column (DE-52) of equal volume. This was washed with 4 volumes of lysis buffer and eluted with a gradient of 0−0.4 M KCl. Fractions containing YDJ-1 were identified by gel electrophoresis. These were pooled, dialyzed against 5 mM potassium phosphate, pH 7.0, 5 mM DTT, and loaded on a column of hydroxylapatite. After washing, the column was eluted with a 5−500 mM phosphate gradient. Fractions containing

YDJ-1 were pooled and concentrated by vacuum dialysis against 10 mM Tris, pH 7.5, 10 mM DTT, 50 mM KCl, and 10% glycerol. Aliquots were stored at −70 °C.

Geldanamycin Treatment. GA was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. GA was dissolved in dimethyl sulfoxide (DMSO) and used at the concentrations indicated.

Antibodies. Mouse monoclonal antibody D7α was prepared against hsp90 purified from chicken brain (Brugge et al., 1983); D7α cross-reacts with native rabbit hsp90. AC88 was prepared against *Achlya* hsp90 and recognizes only free hsp90 (Sullivan et al., 1985). 4F3 was prepared against chicken hsp90, and recognizes both free and complexed hsp90 (Riehl et al., 1985). BB70 was prepared against chicken hsp70 complexed with hsp90 (Smith et al., 1993) and cross-reacts with rabbit hsp70.

HDJ-1 Antiserum. HDJ-1 antiserum was prepared by W. J. Hansen and W. J. Welch as follows. The recombinant HDJ-1 (hsp40) used as an antigen was produced by bacterial expression of the hdj1 gene described by Raabe and Manley (1991). The recombinant protein was solubilized from inclusion bodies with 8 M urea, clarified by centrifugation, and dialyzed to remove the urea. HDJ-1 was then SDS−PAGE-purified, mixed with Freund's complete adjuvant, and injected intradermally into the rabbit. The immune response was monitored by Western blots of the recombinant antigen and by recognition of a heat shock inducible ∼38 kDa species in HeLa cells. The species recognized in lysates of heat shocked and radiolabeled HeLa cells was a basic protein as determined by nonequilibrium pH gradient gel electrophoresis, in agreement with the predicted amino acid sequence.

Immune-Depletion of Hsp90 and DnaJ Proteins. Antibody resins were prepared by incubating antibody with a slurry of protein A−Sepharose CL-4B in 0.1 M potassium phosphate, pH 7.5, for 40 min at room temperature. Typical proportions for DnaJ immune-depletions were 10 μL (packed volume) of resin and 10 μL of rabbit antiserum (prepared against human HDJ-1) or 20 μg of PR22. Antibody−protein A resins were washed once with 0.1 M potassium phosphate and 4 times with TB. Antibody−protein A resins (25 μL) were added to 70 μL of P90 (70 μg of hsp90) and incubated at 4 °C with gentle rocking for 2 h. Samples were pelletted, and the resulting supernatants were treated 2 more times with antibody−protein A resins. Aliquots were saved after each antibody treatment and analyzed for DnaJ proteins and chaperoning activity. For immune-depletion of hsp90 from P90, one AC88 pellet (40 μg of AC88/10 μL of protein A−Sepharose) and two pellets with 4F3 (40 μg of 4F3/10 μL protein A−Sepharose) and D7α (5 μL of ascites/10 μL of protein A−Sepharose) were prepared as above. P90 (150 μg of hsp90) was first incubated with the AC88−protein A pellet (100 μL), and the supernatant was incubated once each with 100 μL of the 4F3/D7α−protein A resins.

Electrophoresis and Western Analysis. Proteins were separated on 10% discontinuous polyacrylamide gels using the procedure of Laemmli (1970) and stained with Coomassie Brilliant Blue R-250. For Western blotting, SDS−PAGE gels were transferred to poly(vinylidene difluoride) membranes (Immobilon-P, Millipore, Bedford, MA) as described elsewhere (Kost et al., 1989), and these were incubated at room temperature for 2−4 h with primary antibodies and for 1 h with alkaline phosphatase-conjugated second antibodies.

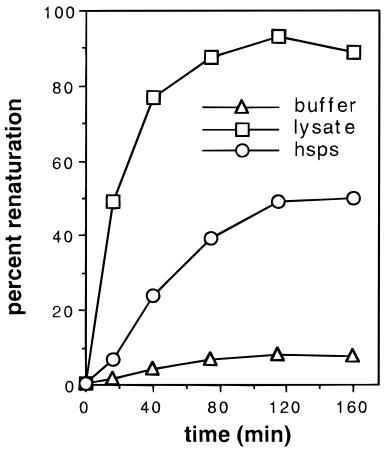

FIGURE 1: Luciferase renaturation in rabbit reticulocyte lysate and hsp preparations. Thermally denatured luciferase (at 100 nM) was diluted 10-fold into buffer (triangles), rabbit reticulocyte lysate (squares), or a heat shock protein preparation (circles). The hsp70 concentration in the hsp sample was 1.4 μM, and the hsp90 concentration (calculated as a dimer) was 275 nM. Luciferase activity was measured at the indicated times, and the percent of luciferase renatured was determined.

RESULTS

The renaturation of thermally denatured firefly luciferase in reticulocyte lysate is very efficient with respect to both its rate ($t_{1/2}$ < 15 min) and its overall recovery (>80% renaturation) (Schumacher et al., 1994). As indicated in Figure 1, renaturation by purified protein samples containing optimal amounts of both hsp70 and hsp90 is much less efficient than renaturation by whole reticulocyte lysate, especially in regard to the initial rate of renaturation. For these experiments, hsp70 and hsp90 were prepared with purities of approximately 85% and 70%, respectively. However, variations of activity of up to 2-fold were observed among different preparations. Therefore, considering the variability of renaturation by hsp preparations and the presence of potentially stimulating factors, our first step was to confirm the roles of hsp70 and hsp90 in the protein folding system, followed by attempts to identify other factors that enhance the extent and initial rate of renaturation.

Hsp70 Is Required for Renaturation. The hsp70 preparations used in most renaturation assays, such as the one shown in Figure 1, are highly purified but not completely free of other proteins. To verify that hsp70 itself was required for this process, we used several recombinant human hsp70 proteins. These hsp70 mutants, which have changes in their final four carboxyl-terminal amino acids (EEVD in wild-type hsp70), have been used previously (Freeman et al., 1995) and have been characterized with respect to their ability to bind native and non-native proteins, their overall conformation, their ability to hydrolyze ATP and the ability of the DnaJ homolog, HDJ-1 (hsp40), to stimulate this hydrolysis, and their ability to refold guanidinium-denatured luciferase. Briefly, the AEVD mutant (an alanine substituted for a glutamic acid) is very similar to wild-type hsp70 in all of these respects. However, the ΔEEVD (deletion of the four carboxyl-terminal amino acids) and AAAA (alanine substitutions) mutants have reduced ability to bind denatured

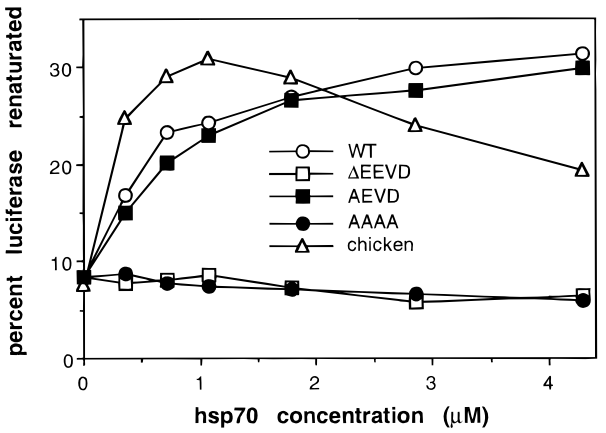

FIGURE 2: Luciferase renaturation using mutant human hsp70 proteins. Thermally denatured luciferase was diluted 10-fold into samples containing chicken P90 (0.85 μM hsp90) and the indicated concentrations of chicken hsp70 (triangles), wild-type human hsp70 (open circles), ΔEEVD human hsp70 (open squares), AEVD human hsp70 (filled squares), and AAAA human hsp70 (filled circles). Luciferase activity was measured after 60 min of renaturation, and the percent of luciferase renatured was determined.

proteins, a higher than normal ATPase activity that is not stimulated by HDJ-1, and an inability to refold guanidinium-denatured luciferase in the presence of ATP and HDJ-1. Figure 2 shows that these same two mutants were also unable to renaturate thermally denatured luciferase in the presence of P90 (chicken hsp90 preparation) while wild-type hsp70 and the AEVD mutant were able to renature ~30% of luciferase. This indicates that a fully functional hsp70 is required for renaturation of thermally denatured luciferase.

Hsp70 and DnaJ Proteins Renature Luciferase in the Absence of Hsp90. We occasionally found that some preparations of hsp70 were able to renature luciferase in the absence of the hsp90 fraction even though these preparations were greater than 85% pure after chromatography upon DEAE-cellulose and ATP−agarose. The renaturing activity was still dependent on ATP, and, although these preparations were free of hsp90, the addition of hsp90 did not further stimulate their renaturing activity (data not shown). In order to separate hsp70 from a potential hsp70-stimulating factor, exceptionally high activity hsp70 preparations were further purified by fractionation on a Superdex 200 sizing column. After fractions were analyzed by SDS−PAGE and stained with Coomassie Blue (Figure 3A), it was apparent that, although the largest portion of hsp70 eluted in the fractions corresponding to monomeric hsp70 (fractions 17−19), much of the hsp70 was in fractions corresponding to higher molecular weight proteins, including void volume fractions (this Superdex 200 column has an exclusion limit of ~600 kDa). This pattern of elution suggests either that some of the hsp70 is polymerized or that it is associated with other proteins.

To assess the effect of Superdex 200 fractionation on chaperoning activity, the hsp70-containing fractions were adjusted to equivalent hsp70 concentrations and assayed for their ability to renature luciferase. To simplify analysis, the fractions were grouped into four pools: pool A included fractions 7−10 and would therefore include protein complexes greater than approximately 425 kDa; pool B included fractions 11−13 (210−425 kDa); pool C included fractions 14−16 (100−210 kDa); and pool D included fractions 17−20 (40−100 kDa). The original hsp70, prior to Superdex 200 treatment, was able to renature over 60% of the denatured luciferase (Figure 3B) in the absence of hsp90. Pools A and B were also able to renature luciferase very effectively while pool C was less efficient but still able to renature some luciferase. Pool D, which would include the monomeric hsp70, was incapable of renaturing luciferase in the absence of other factors. However, the hsp70 in pool D was still functional as indicated by its ability to renature luciferase when P90 was added (data not shown). These results suggest that the activity of hsp70 is influenced by other factors or by its molecular state. A recent report (King et al., 1995) suggests that a DnaJ homolog, YDJ-1, can induce the polymerization of bovine and yeast hsp70 in the presence of ATP but that very little YDJ-1 is part of these higher molecular weight hsp70 complexes. Also, as noted previously, human hsp70 and HDJ-1 together can renature chemically denatured luciferase (Freeman et al., 1995).

To test for the presence of DnaJ proteins in hsp70 fractions resulting from Superdex 200 fractionation, we used a rabbit antiserum prepared against human HDJ-1. On a Western blot with the original hsp70 sample, this antiserum detected four protein bands in the 40−50 kDa range (Figure 3C). These are likely to be DnaJ homologs; however, we have not confirmed their identities by other means. After fractionation on the Superdex 200 column, the only significant DnaJ bands were in fractions 8−12, which correspond to protein molecular masses greater than 250 kDa. Two very light DnaJ bands are present in fractions 16 and 17 where DnaJ would normally elute as a dimer. This suggests that DnaJ proteins are interacting with hsp70 to form higher molecular mass complexes, although other explanations cannot be ruled out. These data do show that fractions with the least amount of DnaJ proteins support the lowest level of protein renaturation.

To determine if DnaJ proteins could stimulate hsp70's renaturation activity in this system, we added YDJ-1 (MAS5), a yeast DnaJ homolog, to the hsp70 fractions obtained from the Superdex 200 column. YDJ-1 caused only a very slight enhancement of renaturation activity by hsp70 that had not been fractionated on the sizing column or hsp70 fractions that eluted in the void volume and highest molecular mass fractions (pool A, Figure 3D). However, YDJ-1 did stimulate renaturation in pools B, C, and D, which correspond to lower molecular mass complexes. Furthermore, the extent of stimulation by YDJ-1 was generally highest in fractions that contain the least amount of endogenous DnaJ proteins (samples C and D). This correlation strongly implicates DnaJ proteins as the factors that activate hsp70 for renaturation of thermally denatured proteins.

Renaturation with fractionated hsp70 in the presence and absence of YDJ-1 suggests that renaturation is quite sensitive to the concentration of DnaJ proteins. We used hsp70 that had fractionated as a monomer on the Superdex 200 column, and was therefore free of endogenous DnaJ proteins, to determine the concentration of YDJ-1 that is optimal for luciferase renaturation. Figure 3E shows that the amount of luciferase renatured using 1.4 μM hsp70 and 10 nM luciferase is greatest when the YDJ-1 concentration is between about 40 and 100 nM. This relatively low YDJ-1 requirement is consistent with the substoichiometric amounts of DnaJ protein in hsp70 preparations. Higher concentrations of YDJ-1 resulted in decreased renaturation activity. The

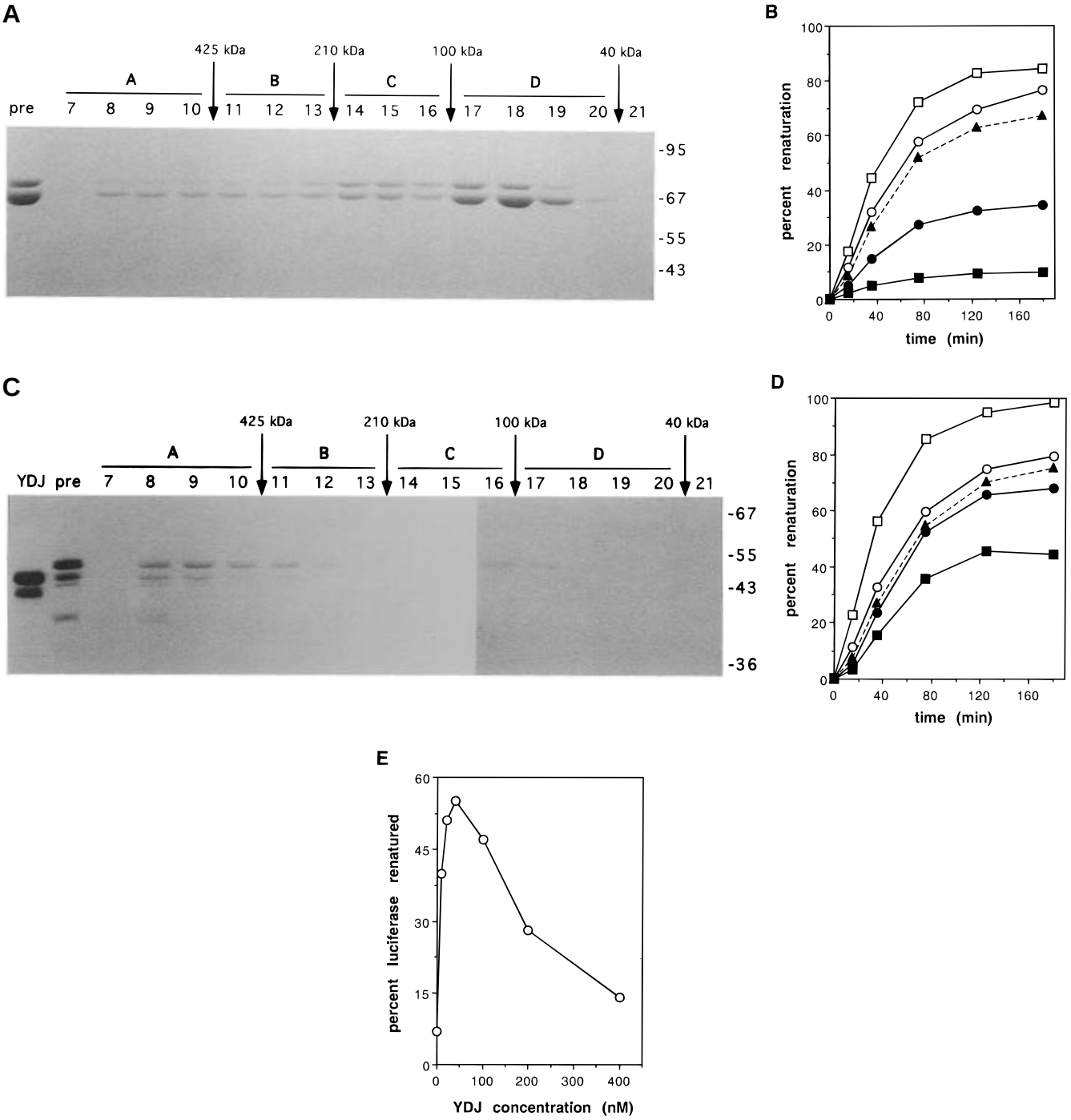

FIGURE 3: Identification of DnaJ proteins in some hsp70 preparations and stimulation by YDJ-1. Chicken hsp70 (~1 mg, ~85% pure) was applied to a Superdex 200 sizing column equilibrated in TB (10 mM Tris, pH 7.5, 3 mM $MgCl_2$, 50 mM KCl, and 1 mM DTT). The column was run at 1 mL/min, and 3 mL fractions were collected. (A) Fifty microliters of the indicated fractions was analyzed by SDS−PAGE and visualized with Coomassie Blue. An aliquot of the starting material (~5 μg) is shown in the first lane (pre), and molecular mass standards are shown on the right. The major band above hsp70 has been shown to be Bip by sequence analysis. The fractions were later grouped into four pools (A−D), and these are indicated above the gel. The approximate molecular mass range of each pool is also indicated. (B) Pooled Superdex 200 fractions were analyzed for chaperoning activity using the luciferase renaturation assay. The hsp70 concentration in each assay was 1.4 μM. No other proteins were added. The samples were original sample before fractionation (triangles), Superdex pool A (open circles), Superdex pool B (open squares), Superdex pool C (filled circles), and Superdex pool D (filled squares). (C) Superdex 200 fractions (80 μL) were separated by SDS−PAGE, transferred to Immobilon, and Western-blotted for DnaJ proteins using a rabbit antiserum prepared against HDJ-1. Bacterially expressed YDJ-1 (250 ng) was used as a positive control. Molecular mass standards are indicated on the right. Pooled fractions are also indicated above. (D) Pooled fractions were analyzed for chaperoning activity as they were in panel B except 40 nM YDJ-1 was added to each sample. The samples were original sample before fractionation (triangles), Superdex pool A (open circles), Superdex pool B (open squares), Superdex pool C (filled circles), and Superdex pool D (filled squares). (E) The optimal concentration of YDJ-1 was determined using hsp70 (1.4 μM) that was free of endogenous DnaJ proteins. The percent of luciferase renatured was determined after 180 min of renaturation.

reason for the inhibitory effect of YDJ-1 at higher concentrations is unknown. Because even low levels of DnaJ proteins can affect hsp70's activity in luciferase renaturation, all further studies reported here were performed with hsp70 that had been additionally purified on the Superdex 200 column and were free of DnaJ proteins based on Western analysis.

Since the yeast DnaJ homolog was able to support renaturation in the presence of hsp70, we also tested a human DnaJ homolog, HDJ-1. This DnaJ protein lacks 2 domains that are conserved in both *E. coli* DnaJ and YDJ-1: an approximately 35 amino acid region that is rich in glycine and phenylalanine, and a cysteine-rich region of approximately 150 amino acids (Cyr et al., 1994). In this system, HDJ-1 was only about 30% as effective as YDJ-1 over the entire concentration range tested (data not shown).

Hsp70 and Hsp90 Cannot Support Renaturation in the Absence of DnaJ Proteins. As indicated above, chicken and rabbit reticulocyte lysate hsp90 preparations (P90) were able to renature luciferase in the presence of hsp70 (Schumacher et al., 1994). However, the variability of the P90's contribution to renaturation suggested that factors other than hsp90 may be involved. We used hsp90 antibodies to remove hsp90 from P90 preparations, and then analyzed the remaining fraction for its ability to stimulate luciferase renaturation. Three hsp90 antibodies, AC88, D7α, and 4F3, were used in these immune-depletion experiments to ensure the most efficient removal of hsp90. The antibodies were adsorbed to protein A−Sepharose, and P90 was incubated first with bound AC88 and then incubated twice with a combination of D7α and 4F3. By this method, more than 90% of the hsp90 was removed from P90 preparations, and in some experiments, no hsp90 remained as determined by Coomassie Blue staining of polyacrylamide gels (see Figure 4B). These hsp90-depleted P90 fractions, as well as untreated P90 samples, were then analyzed for their ability to stimulate renaturation of luciferase in the presence of hsp70. As shown in Figure 4A, the hsp90-depleted P90 sample (antibody-treated P90) still retained most of its ability to stimulate luciferase renaturation when combined with hsp70. Furthermore, a preparation of human hsp90 (Hhsp90) that was about 95% pure caused only a small increase in renaturing activity under these conditions (Figure 4A). Collectively, these data show that hsp70 and hsp90 cannot support renaturation and that there is another factor found in some preparations of P90 that is able to stimulate the renaturation of luciferase by hsp70.

DnaJ Proteins Are Stimulatory Factors in P90 Preparations. The above experiments demonstrate that YDJ-1, chicken DnaJ proteins, and factors other than hsp90 are able to stimulate hsp70's ability to renature thermally denatured luciferase. We next used the rabbit antiserum prepared against human HDJ-1 to test whether DnaJ proteins were present in P90 preparations. Western analysis with this antiserum revealed five protein bands (Figure 5A, lane 2), indicating the presence of DnaJ proteins in the P90 preparation. However, no DnaJ protein bands were present in the highly purified human hsp90 (Hhsp90, lane 9). To determine whether these DnaJ proteins were also the stimulating factors present in P90 preparations, we treated high-activity P90 preparations with HDJ-1 antiserum to remove any potential DnaJ proteins. Hsp90 preparations were treated 3 times with anti-DnaJ antibodies bound to protein A−Sepharose. PR22, an antibody to the progesterone receptor, was used in control experiments. After antibody treatment, the hsp90 samples were analyzed for the presence of DnaJ proteins and for their ability to stimulate hsp70's renaturing activity (Figure 5). Western analysis indicated that each anti-DnaJ antibody treatment decreased the DnaJ protein levels in hsp90 preparations while treatment with PR22 did not (Figure 5A).

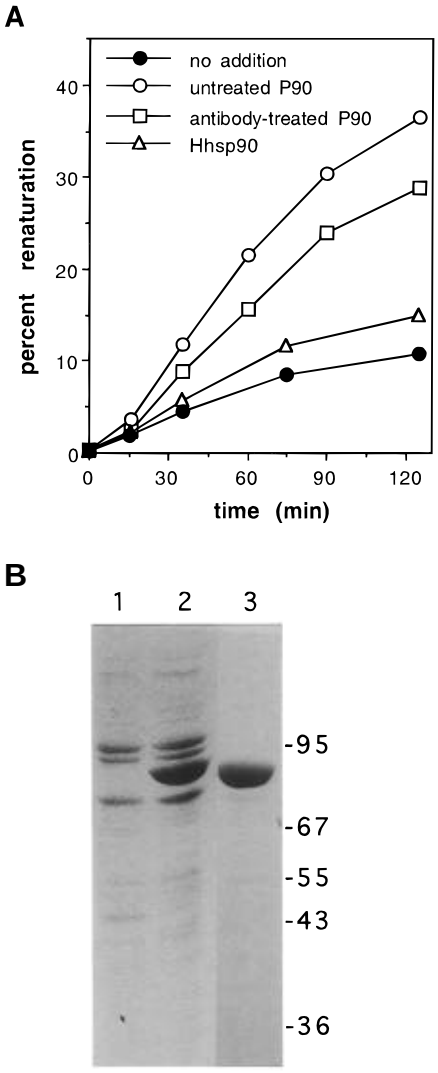

FIGURE 4: Non-hsp90 stimulating factors in P90 preparations. Hsp90 was removed from a chicken P90 preparation by antibody adsorption as described under Results. (A) To analyze for chaperoning activity, the following additions were made to samples containing 1.4 μM DnaJ−free hsp70: no addition (filled circles), P90 before (circles) and after (squares) immune-depletion of hsp90, and the more highly purified human hsp90 (Hhsp90, triangles). The hsp90 concentration in the untreated P90 and Hhsp90 samples was 275 nM. (B) P90 and Hhsp90 were separated by SDS−PAGE and stained with Coomassie Blue. Lane 1, P90 after immune-depletion of hsp90; lane 2, P90 before immune-depletion of hsp90; lane 3, Hhsp90. Molecular weight standards are indicated on the right.

Additionally, while P90 preparations initially had very high stimulating activity, each successive DnaJ antibody treatment decreased the P90 preparations ability to stimulate renaturation (Figure 5B). To provide additional evidence that DnaJ proteins were the stimulating factors in P90 preparations, we added YDJ-1 back to P90 that had been treated with DnaJ antibodies (data not shown). YDJ-1 did not further stimulate untreated P90 or P90 that had been treated with PR22. However, the addition of YDJ-1 to P90 that had been treated with anti-DnaJ antibodies increased renaturation to levels very near those observed with untreated P90. These results show that the major contribution to renaturation in the P90 fraction is not hsp90 but rather DnaJ homologs that contaminate this fraction.

Activity of Hsp90 in Renaturation. The demonstration that the above DnaJ-related factors can support the renaturation of luciferase in the presence of hsp70 suggests a lesser role,

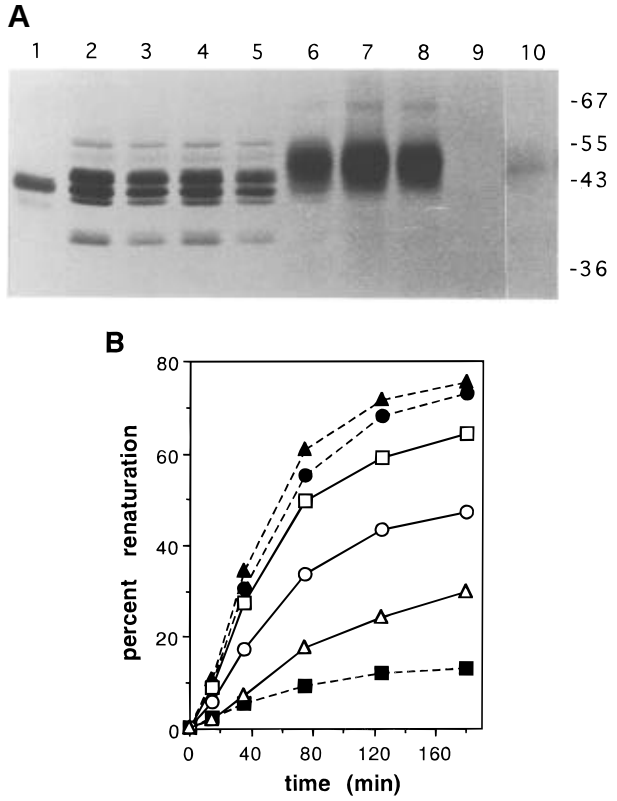

FIGURE 5: Immune-depletion of DnaJ proteins from P90. DnaJ proteins were immune-depleted from a chicken P90 preparation as described under Results. (A) Samples were separated by SDS−PAGE, transferred to Immobilon, and Western-blotted for DnaJ proteins using the rabbit antiserum prepared against HDJ-1. Lane 1, 100 ng of YDJ-1; lane 2, 5.3 μL of P90 before immune-depletion of DnaJ proteins; lanes 3−5, 5.3 μL of P90 after 1, 2, and 3 immune-depletions, respectively, with control antibody (PR22); lanes 6−8, 5.3 μL of P90 after 1, 2, and 3 immune-depletions, respectively, with anti-DnaJ antiserum; lane 9, 2.6 μg of Hhsp90; lane 10, P90 with no primary antibody. Molecular weight standards are indicated on the right. (B) P90 samples were analyzed for chaperoning activity after immune-depletions. All samples had 1.4 μM DnaJ−free hsp70 and 3.5 μL of the indicated P90 sample (275 nM hsp90). The samples were without P90 (filled squares), with untreated P90 (filled circles), with P90 treated twice with control PR22 antibody (filled triangles), and with P90 treated with anti-DnaJ antiserum 1 (open squares), 2 (open circles), or three 3 (open triangles).

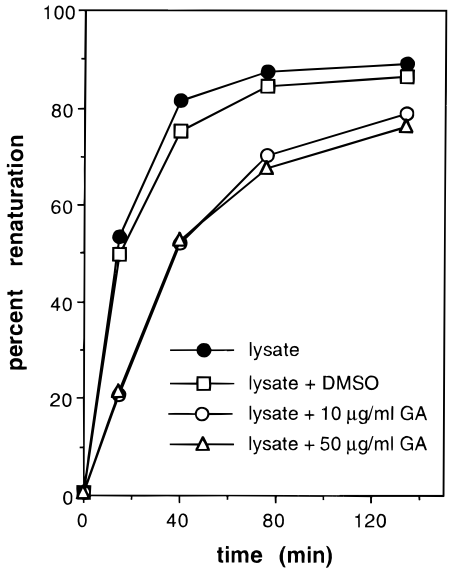

FIGURE 6: Hsp90-binding GA partially inhibits renaturation. Reticulocyte lysate was assayed for renaturation activity after the following treatments: none (filled circles); GA solvent, DMSO (squares); 10 μg/mL GA (open circles); and 50 μg/mL GA (triangles).

if any, for hsp90 in the renaturation of thermally denatured proteins. However, further studies demonstrate that hsp90 is definitely able to assist hsp70 in protein renaturation. This was tested by use of highly purified hsp90 (that was free of contaminating DnaJ proteins) and the hsp90 inhibitor geldanamycin. Geldanamycin (GA), a benzoquinone ansamycin, has been shown to bind directly to hsp90 (Whitesell et al., 1994) and disrupt hsp90's association with $pp60^{v-src}$ (Whitesell et al., 1994) and raf-1 (Schulte et al., 1995), as well as with the hsp90-binding protein p23 (Johnson & Toft, 1995). We therefore tested whether GA would affect hsp90's activity in the renaturation of luciferase. We first analyzed the effect of GA on luciferase renaturation in reticulocyte lysate (Figure 6). In the absence of GA, reticulocyte lysate renatures almost 90% of luciferase with a half-time of <15 min. In the presence of 10 μg/mL GA, almost 80% of the luciferase is renatured, but the half-time of renaturation is approximately doubled. This concentration of GA is consistent with those used to inhibit other hsp90 activities (Whitesell et al., 1994; Johnson & Toft, 1995), and higher concentrations (50 μg/mL) do not further inhibit renaturation (Figure 6). Furthermore, when GA is added to samples containing P90 and hsp70, renaturation is inhibited almost to the same degree as when hsp90 is immune-depleted from P90 (data not shown). This inhibition indicates a role for hsp90 in renaturation of luciferase in whole reticulocyte lysate.

The stimulating components in the P90 preparations appear to be hsp90 and DnaJ proteins. The availability of YDJ-1 and highly purified hsp90 allowed us to investigate directly any possible role for hsp90 in assisting hsp70 and DnaJ proteins in renaturation. Hhsp90 was not able to stimulate renaturation with hsp70 in the absence of other factors (Figure 4A). Figure 7A shows that at optimal YDJ-1 concentrations Hhsp90 had only a slight positive effect on protein renaturation. However, at YDJ-1 concentrations above the optimum, where renaturation was limited, the addition of Hhsp90 allowed significantly more renaturation. Furthermore, the maximal renaturation in the presence of Hhsp90 was better than renaturation with only hsp70 and any concentration of YDJ-1, suggesting that hsp90 was doing more than simply dampening the inhibitory effects of YDJ-1 at higher concentrations. To investigate this further, we added GA to samples containing Hhsp90 and YDJ-1 concentrations that showed the greatest hsp90 stimulation (Figure 7B). With this high YDJ-1 concentration (160 nM), the renaturation of luciferase is minimal in the absence of hsp90, but is very substantial in its presence. Treatment with GA did not inhibit the slight renaturation by hsp70 and YDJ-1, but did inhibit 30−50% of the hsp90 stimulation. These findings suggest that the contribution of hsp90 may be complex with both a GA-sensitive and an insensitive activity.

After demonstrating that hsp90 can participate in the renaturation of thermally damaged luciferase, we further investigated the role of hsp90 in this process. To do this, we began the renaturation process in the absence of hsp90 and then added hsp90 at different times throughout the renaturation process (Figure 8). Under these conditions (120 nM YDJ-1, 800 nM hsp70, and no hsp90), YDJ-1 and hsp70

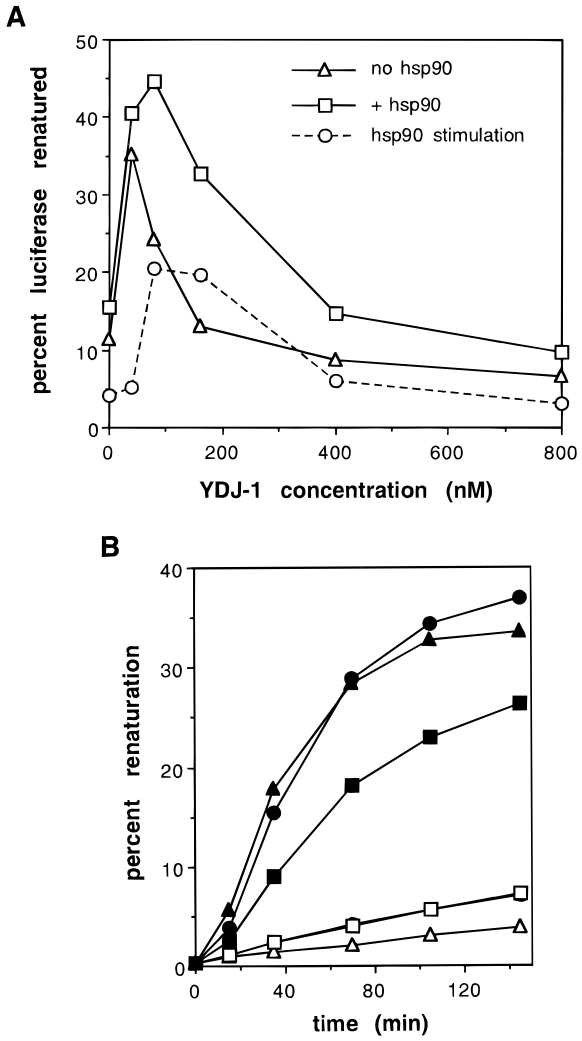

FIGURE 7: Hsp90 stimulates renaturation by hsp70 and YDJ-1. (A) Luciferase renaturation was measured after the addition of the indicated concentration of YDJ-1 to 1.4 μM DnaJ-free hsp70 in the absence (triangles) or presence (squares) of 275 nM Hhsp90. The difference in the percent of luciferase renatured in the presence and absence of Hhsp90 is also shown (circles). Luciferase activity was measured after 140 min of renaturation. (B) Measurement of luciferase renaturation by 400 nM hsp70 and 160 nM YDJ-1 in the absence (open symbols) and presence (closed symbols) of 500 nM Hhsp90 after the following additions: no additions (triangles); DMSO (circles); and 10 μg/mL GA (squares).

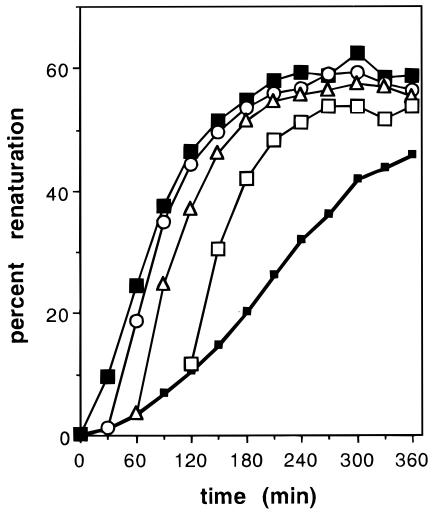

FIGURE 8: Hsp90 stimulates renaturation even after delayed addition. Luciferase renaturation was measured at 25 °C in the presence of hsp70 (800 nM) and YDJ-1 (120 nM). At the indicated times (0, 30, 60, and 120 min), aliquots of renaturing samples were withdrawn, 300 nM Hhsp90 was added, and renaturation at 25 °C was continued.

can renature more than 40% of the luciferase after 6 h but the renaturation is very slow. Addition of Hhsp90 at the beginning of the renaturation allows much more efficient renaturation, similar to the results shown in Figure 7. Furthermore, the delayed addition of hsp90 still stimulates renaturation in a manner similar to that observed when hsp90 was added at the start of renaturation. Two slight differences are apparent, however. First, noticeably less luciferase is renatured when hsp90 is added 120 min after the initiation of renaturation. Also, when hsp90 is added after 30, 60, or 120 min, the renaturation proceeds without the slight, but consistent, lag observed when hsp70, YDJ-1, and hsp90 are combined at the initiation of renaturation.

DISCUSSION

The main objectives of this study were to define the components required for the renaturation of thermally denatured firefly luciferase in cytosol extracts and to initiate a search for factors that influence this renaturation activity. We had previously reported that samples containing hsp70 and hsp90 could support some renaturation of thermally denatured luciferase. However, it is now clear that both of these proteins, purified by common methods, were frequently contaminated by low, but very effective, levels of DnaJ proteins. These results emphasize the importance of using chaperone proteins of high purity. We have now found that this system is most dependent on hsp70 and DnaJ proteins. The requirement for hsp70 and DnaJ proteins for protein renaturation in the eukaryotic cytoplasm is consistent with other recent work concerning protein folding in bacteria and eukaryotes (Lanager et al., 1992; Schröder et al., 1993; Frydman et al., 1994; Freeman et al., 1995; Hendrick et al., 1993; Skowyra et al., 1990; Kudlicki et al., 1995; Ziemienowicz et al., 1995). Hsp90 was not an essential component of this renaturation system; however, it could enhance the renaturation process particularly when using higher than optimal concentrations of the other two chaperones. Furthermore, partial inhibition of hsp90 activity slowed luciferase renaturation even in the very efficient chaperoning environment of reticulocyte lysate, suggesting that hsp90 is an important component for renaturation in vivo.

In eukaryotes, the study of hsp70 and DnaJ function is complicated by the presence of numerous compartmental homologs that may have developed specialized functional roles. The mammalian cytoplasm contains two major hsp70 proteins, and yeast have at least six cytoplasmic hsp70s which belong to two distinct families (Rassow et al., 1995). DnaJ proteins have been identified in all eukaryotic compartments that contain hsp70 (Cyr & Douglas, 1994). Four human DnaJ homologs have been cloned (HDJ-1, HDJ-2, HSJ-1a, and HSJ-1b) while yeast have at least two DnaJ homologs that are localized to the cytoplasm, YDJ-1 and the less abundant SIS-1, and several DnaJ homologs in various organelles (Cyr et al., 1994; Schlenstedt et al., 1995; Rowley et al., 1994).

Our Western analysis with the HDJ-1 antiserum suggests that multiple DnaJ homologs exist in cell lysates. Although

this antiserum was prepared against HDJ-1, it also recognizes HDJ-2 (W. J. Hansen and W. J. Welch, unpublished observation). We have shown that in a purified system, the yeast DnaJ homolog, YDJ-1, can replace the activity of some of these DnaJ homologs in protein renaturation while HDJ-1 does so very poorly. We have isolated a fraction from rabbit reticulocyte lysate that is free of hsp70 and hsp90, but contains multiple DnaJ homologs that potently stimulate renaturation in the presence of hsp70 (results not shown). Western blotting of this preparation indicates the presence of homologs to both HDJ-1 and HDJ-2. HDJ-2 is more homologous to YDJ-1 in domain structure, and it is possible that this protein, or the combination of DnaJ proteins, causes the potent stimulation of renaturation. To fully understand protein renaturation in the eukaryotic cytoplasm, the various DnaJ homologs present in the cytoplasm need to be isolated and characterized biochemically.

Our studies using purified proteins indicate that little DnaJ is actually needed to stimulate hsp70 chaperoning activity. This is evident both with the endogenous DnaJ proteins and with YDJ-1. For the data shown here, where the hsp70 concentration is typically 1.4 μM, the optimal YDJ-1 concentration is about 40 nM (see Figure 3E), yielding an hsp70:YDJ-1 ratio of 35:1. In related experiments, we have found that with 10 nM luciferase and 40 nM YDJ-1 the optimal concentration of hsp70 is actually closer to 400 nM (renaturation is equally efficient when the luciferase concentration is 50 nM, and the luciferase:hsp70:YDJ-1 ratio is 1:4:1; data not shown). These substoichiometric concentrations of YDJ-1, relative to hsp70, are in agreement with results from other groups who have shown that *E. coli* DnaJ proteins can catalytically activate DnaK's binding activity and its ATPase activity (Liberek et al., 1995), and that YDJ-1 can catalytically induce the polymerization of yeast and bovine hsp70 in an ATP-dependent process (King et al., 1995). It should be noted that our assay system contains glycerol (1%) and Triton (0.025%) which minimize the aggregation of denatured luciferase (Schumacher et al., 1994). This may reduce the requirement for DnaJ proteins if they play a role in preventing aggregation, as reported by Cyr (1995).

Although hsp70 and DnaJ appear to be the most important proteins for renaturation in reticulocyte lysates, this work demonstrates that hsp90 also contributes to protein renaturation. This was clarified by the use of highly purified hsp90 that was not contaminated with other factors, and by the use of geldanamycin, a specific inhibitor of hsp90 function. Previous studies have shown that GA is able to disrupt hsp90's association with $pp60^{v-src}$ (Whitesell et al., 1994), raf-1 (Schulte et al., 1995), and the hsp90-binding protein p23 (Johnson & Toft, 1995). It also modifies the interaction of hsp90 with progesterone receptor (Johnson & Toft, 1995; Smith et al., 1995), and it has been used to indicate the association of hsp90 with mutated p53 (Blagosklonny et al., 1995) and the reverse transcriptase of hepatitis B virus (Hu & Seeger, 1996). However, this is the first use of GA in protein renaturation studies, and it is also the first demonstration that GA does not affect the chaperoning activities of hsp70 or DnaJ proteins.

The addition of GA to reticulocyte lysate reduces the renaturation rate by half, indicating an effect of hsp90 early in the renaturation process. However, in the purified system, the effects of GA are more evident throughout the time course of renaturation. This is consistent with the observation that hsp90 is still very effective when added long after the initiation of the renaturation process. Not surprisingly, the hsp90 stimulation resulting from delayed addition of hsp90 is also partially GA-sensitive (data not shown). The partial inhibition of hsp90 by GA in the purified system suggests that hsp90 may act in more than one fashion to enhance the chaperoning activity.

Further studies are needed to define the role of hsp90 in this system since several possibilities exist. Previous studies have shown that hsp90 alone can stabilize several proteins as folding intermediates to maintain them in a nonaggregated state, capable of refolding to native protein (Jacob et al., 1995; Freeman & Morimoto, 1996; Yonehara et al., 1996). This effect of hsp90 did not require ATP or other proteins. On the other hand, the association of hsp90 with steroid receptors has been shown to be a complex process requiring ATP hydrolysis and several additional proteins (Smith et al., 1992; Johnson & Toft, 1994, 1995). While hsp90 has been reported to bind ATP, this property has been disputed in recent studies (Jakob et al., 1996). In the present study, hsp90 may be stabilizing folding intermediates of luciferase, as has been observed in other systems. However, the delayed addition experiments suggest that if this is the case, hsp90 stabilizes intermediates downstream of their interaction with hsp70 and/or YDJ-1. Hsp90 may also interact with hsp70 and/or YDJ-1 to modify their activities, particularly when these proteins are in excess. It is also possible that hsp90 has an ATP-dependent chaperoning activity, but this remains to be established. The partial sensitivity to GA suggests that more than one of these hsp90 activities is involved throughout the cooperative renaturation process.

The present study provides clarification on the proteins essential for the recovery of thermally damaged proteins in the eukaryotic cytoplasm. It also indicates that additional factors are needed for optimal chaperoning activity. Hsp90 appears to be one such factor; the comparison with whole lysate suggests that there are more. This assay provides a means for identifying these factors and for describing their individual contributions.

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