We have developed an assay for chaperone-mediated protein renaturation using thermally denatured Firefly luciferase. Dilution of denatured luciferase (>99% loss of activity) into reticulocyte lysate typically results in recovery of 5-15% activity. Addition of an ATP-regenerating system increases yields to >60%, while heat shock or the addition of denatured proteins inhibits the chaperoning activity. Reticulocyte lysate contains abundant quantities of the heat shock proteins, hsp90 and hsp70, and a 60-kDa protein homologous to the yeast stress protein, STI1. Immune isolated samples of these three proteins support recovery of up to 35% of luciferase activity in an ATP-dependent manner, suggesting that these or associated proteins are involved in the renaturation of luciferase. Furthermore, we observed a correlation between luciferase renaturation activity and the levels of hsp70 and hsp90 in reticulocyte lysate preparations. Purified hsp90 and hsp70, along with an ATP-regenerating system, are able to renature luciferase to greater than 20% of its original activity. This renaturation is most efficient when hsp90 and hsp70 are at about a 2:1 ratio and at concentrations similar to those found in reticulocyte lysate. This study provides evidence for an ATP-dependent chaperoning activity in reticulocyte lysate that involves a cooperative action of hsp70 and hsp90.

It is now apparent that protein folding in the cell is more complicated than previously thought. Protein folding in the mitochondria, endoplasmic reticulum, and cytoplasm is likely to be mediated by a group of proteins called molecular chaperones (1, 2). Although many of these proteins were first identified as heat shock proteins (hsp), most are also constitutively expressed. These proteins function as molecular chaperones in vivo to recognize and stabilize partially folded intermediates during polypeptide folding, assembly, and disassembly (1, 2). Many molecular chaperones belong to one of three highly conserved families, hsp90, hsp70, and hsp60 (also called chaperonins).

Chaperonins (Escherichia coli GroEL, mitochondrial hsp60, and chloroplast Rubisco-binding protein) have a characteristic oligomeric structure consisting of two stacked heptameric rings of ∼60-kDa subunits (3-5), while the hsp70s (including the E. coli homologue DnaK) probably act as monomers or dimers. Although these two families are structurally unrelated, both require ATP hydrolysis for release of bound protein substrate. Recent evidence suggests that chaperonins, hsp70s, and additional proteins can function cooperatively in a sequential folding pathway (6-8). In bacteria, DnaK cooperates with two additional stress proteins, DnaJ and GrpE, to maintain proteins in a partially folded intermediate, which is then presented to GroEL/GroES for folding to the native state (6). Several reports also suggest that similar cooperative folding pathways may exist for proteins transported into the mitochondria (9, 10).

Although there are numerous reports supporting chaperone-mediated protein folding in bacteria and mitochondria, there is much less evidence for these mechanisms in the eukaryotic cytoplasm. Several studies have shown that members of the hsp70 family of chaperones, along with an N-ethylmaleimide-sensitive cystolic factor, are required for the import of proteins into the mitochondria (11-13). hsp70 also binds to newly synthesized polypeptides in the cytoplasm (14, 15) and to denatured proteins in vitro (16). Recently, it was reported that the TCP-1 complex, a eukaryotic cytoplasmic homologue of chaperonin 60, can associate with non-native proteins and mediate their ATP-dependent folding (17-19).

hsp90 is perhaps the most abundant constitutively expressed stress protein in the eukaryotic cytoplasm. The most studied interaction of hsp90 has been with unactivated steroid receptors (20-22). hsp90 is also reported to associate with a diverse range of cellular proteins including the protein kinases, casein kinase II (23), the heme-regulated eIF-2α kinase (24, 25), and pp60src (26), and the cytoskeletal proteins, actin (27) and tubulin (28). The common feature of these associations seems to be stabilization of target proteins in an inactive, partially unfolded, or disassembled state. hsp90 has recently been shown to have peptide-stimulated ATPase activity, although a functional requirement for this activity has not been identified (29). It has previously been demonstrated that bovine hsp90 prevents non-native proteins from unproductive aggregate interactions in an ATP-independent manner (30), and hsp90 has also been implicated in the conformational activation of MyoD (31), suggesting that it may act as a general chaperone.

Rabbit reticulocyte lysate has unique capabilities for in vitro protein synthesis and also contains high levels of molecular chaperones that may be involved in protein folding (32-34). Several laboratories have recently utilized rabbit reticulocyte lysate for analysis of chaperone-mediated folding of proteins such as actin (19), tubulin (18, 35), mitochondrial aspartate aminotransferase (36), and luciferase (37). We and others have used rabbit reticulocyte lysate to study the assembly of steroid receptor complexes, an ATP-dependent process that involves hsp90, hsp70, and additional factors (34, 38, 39). A similar complex involving these proteins also appears to occur with the heme-regulated eIF-2α kinase (25). In an attempt to simplify
the measurement of chaperone activity in reticulocyte lysate, we have developed an assay for chaperoning activity using luciferase as a reporter enzyme. With this assay, we have identified ATP-dependent chaperoning activity in rabbit reticulocyte lysate. This activity is independent of the TCP-1 complex proteins but correlates with the levels of hsp70 and hsp90 present in reticulocyte lysate. We have also shown that hsp90 and hsp70 can at least partially substitute for reticulocyte lysate in this ATP-dependent chaperoning, indicating that these two proteins can function cooperatively in assisting protein folding.

**Experimental Procedures**

Buffers—The buffers used were as follows: Triton buffer (TB) consisting of 10 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, 100 mM KC1, and 2 mM dithiothreitol (DTT); assay buffer (AB) consisting of 25 mM Tricine-HCl, pH 7.75, 8 mM MgSO₄, 0.1 mM EDTA, 12 mM DTT, 100 mM d-luciferin, 240 mM coenzyme A, and 0.5 mM ATP; stability buffer (SB) consisting of 25 mM Tricine-HCl, pH 7.75, 8 mM MgSO₄, 0.1 mM EDTA, 10 mg/ml BSA, 10% glycerol, and 1% Triton X-100.

Luciferase Renaturation Assay—Firefly luciferase (Sigma), in SB at 3.2 × 10⁻¹⁰⁻¹² to 3.2 × 10⁻¹⁰⁻¹³ M, was denatured by incubation at 40 °C. After the luciferase had lost less than 99% of its original activity (10 min) it was diluted 10-fold into the sample to be analyzed for renaturation activity at 30 °C. Twenty-five μl of the sample was then added to 100 μl of AB in a 1 ml Eppendorf tube. The sample was analyzed for luciferase activity by placing the Eppendorf tube in a glass scintillation vial and counting for 1 min on a scintillation counter (Beckman 2801) with all windows open. In all experiments, the activity was expressed as a percent of control samples that were handled identically but without 40 °C treatment. Untreated rabbit reticulocyte lysate was purchased from Green Hector (Oregon, WI) and stored at -70 °C. The ATP-regenerating system used contained 10 mM phosphocreatine (Sigma) and 3.5 μmol of creatine kinase (type I, rabbit muscle, Sigma) 100 μl of sample.

To prepare reticulocyte lysate with different levels of hps, healthy 3-month-old New Zealand White rabbits (1.5-2.5 kg) were injected subcutaneously with 2 mL of 1% body F5 was prepared against gel-purified chicken p60 and cross-reacts with rabbit p59-A cDNA clone for human p60 (protein iEF SSP 3521) was generously supplied by B. Honore, Aarhus University (47). This was placed in a PET-11 expression vector and was overexpressed and purified as described above. After lysis by sonication and centrifugation for 15 min at 10,000 rpm, the supernatant in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10 mM monoothioglycerol was fractionated on DEAE-cellulose by 0–400 mM KC1 gradient elution. Fractions containing p60 were pooled and further fractionated by 10–400 mM phosphate elution on a hydroxyapatite column. The hsp70-containing fractions identified by gel electrophoresis were pooled, concentrated by ammonium sulfate precipitation (75% saturation), and the precipitate was dissolved and dialyzed into TB and stored at -70 °C. The human homologue of DnaJ, HDJ-1, and rabbit p59 was expressed and purified as described above for p60. A cDNA clone for HDJ-1 was kindly provided by T. Raabe and J. L. Manley (48). The p59 expression system was prepared in collaboration with L. E. Faber (49).

Antibodies—Mouse monoclonal antibody D7α was prepared against hsp90 purified from chicken brain (50); D7α cross-reacts with native rabbit hsp90. BB70 was prepared against chicken hsp70 complexed with hsp90 (51) and cross-reacts with rabbit hsp70. Monoclonal antibody F5 was prepared against gel-purified chicken p60 and cross-reacts with p60 from a variety of vertebrates (51).

Immunoprecipitation of Proteins—Antibody-resin were prepared by incubating antibody with a slurry of protein A-Sepharose CL-4B in 100 mM potassium phosphate, pH 8.0, for 30 min at room temperature. Typical proportions were 10 μg of antibody to 10 ml packed volume of resin. Antibody-protein A resin was added to reticulocyte lysate and incubated at 4 °C with gentle rocking for 2 h. Resin was washed four times with 1 ml of TB by suspension and centrifugation and used in luciferase renaturation assays or analyzed by SDS-polyacrylamide gel electrophoresis.

Electrophoresis—Proteins were separated on 10% discontinuous polyacrylamide gels using the procedure of Laemmli (52) and stained with Coomassie Brilliant Blue R-250.

RESULTS

Luciferase Renaturation Assay—An example of a standard luciferase renaturation assay is shown in Fig. 1. Firefly luciferase was first denatured by incubation at 40 °C. After 10 min, luciferase activity was reduced to less than 1% of its original activity. Dilution of this denatured luciferase into buffer at 30 °C did not result in any recovery of luciferase activity. The addition of 2 mM ATP and an ATP-regenerating system to the buffer did not improve luciferase activity, showing that luciferase does not refold to a native state spontaneously under the conditions used. However, the results were quite different when denatured luciferase was incubated in rabbit reticulocyte lysate. Luciferase activity reached 10–20% of the original activity when denatured luciferase was diluted into untreated rabbit reticulocyte lysate at 30 °C. Supplementing the reticulocyte lysate with an ATP-regenerating system increased luciferase activity to near 100%. These results indicate an ATP-dependent activity in reticulocyte lysate that is able to restore luciferase to its native state.

In additional experiments, we found that reticulocyte lysate
Fig. 1. Luciferase denaturation and reactivation. Luciferase was thermally denatured in SB at 40 °C for 10 min and then diluted 10-fold into either fresh buffer (○) or reticulocyte lysate (■ and □) at 30 °C to allow renaturation. Renaturation was in the presence (○ and ■) or absence (□) of 2 mM ATP and an ATP-regenerating system (ATP/RS) as indicated in the key. Luminescence was measured at the indicated times on a scintillation counter for 1 min. Activity of renatured luciferase was compared with the activity of luciferase at the same concentration and temperature, that had not been thermally denatured.

It did not protect luciferase from denaturation at 40 or 42 °C. Furthermore, lysate that was preincubated at 40 (data not shown) or 42 °C (Fig. 2) lost most of its ability to restore luciferase activity at 30 °C. While both hsp70 and hsp90 have been reported to undergo heat-induced changes in conformation, both proteins appear to be stable at 42 °C in the presence of physiological ATP concentrations (16, 53–55). It is currently thought that heat shock induces the accumulation of denatured proteins and protein aggregates, which compete for the binding of hsp70 and effectively reduce the functional pool of hsp70 that is available to interact with other proteins (56). The addition of denatured but not native protein should then lead to the apparent inhibition of the chaperone activity of hsp70 in the absence of denaturing conditions. Denatured RCM-BSA has been shown to bind hsp70, blocking its interaction with the heme-regulated eIF-2α kinase (33). The ability of lysate to restore luciferase activity was inhibited by 20 μM denatured RCM-BSA, while native BSA was found to have no effect (Fig. 2). This observation suggests that the apparent heat-induced inhibition of renaturation activity in lysate is probably due to accumulation of denatured proteins that subsequently compete with the luciferase for the binding of chaperones rather than the thermal instability of the protein activities that are responsible for the renaturation of the luciferase.

Dialyzable Factors Required for Luciferase Renaturation—We next examined reticulocyte lysate for dialyzable factors that may be required for luciferase reactivation. Lysate was dialyzed in 10 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, and 2 mM DTT. Dilution of denatured luciferase into dialyzed reticulocyte lysate did not result in any renaturation over a 45-min period (Fig. 3). The addition of 100 mM KCl did not improve renaturation; however, when ATP and an ATP-regenerating system were added to dialyzed reticulocyte lysate, luciferase activity was restored to greater than 40% of its original activity in less than 60 min. The combination of 100 mM KCl and an ATP-regenerating system supported luciferase recovery of greater than 80%. Thus, KCl and Mg²⁺-ATP appear to be the only dialyzable factors required for efficient renaturation of luciferase in reticulocyte lysate. Reducing agents, such as thioglycerol and DTT, improve renaturation, although they are not required (data not shown).

Sucrose Gradient Fractionation of Reticulocyte Lysate—It is now thought that hsp70 plays a major role in protein folding and renaturation catalyzed in vivo (1, 2). The TCP-1 ring complex has been reported to support ATP-dependent refolding of luciferase (17). This complex sediments in the 19–20 S range on sucrose gradients (17, 57). We performed sucrose gradient experiments on lysate to determine whether the TCP-1 complex might be responsible for the renaturation activity we observed.
Reticulocyte lysate was fractionated on 15–30% linear sucrose gradients (Fig. 4), and fractions were dialyzed in 10 mm Tris-HCl, pH 7.5, 3 mm MgCl₂, 100 mm KCl, and 2 mm DTT. Analysis of luciferase renaturation in these fractions resulted in a peak of activity at 8–10 S with a very low level of activity at 19–20 S (Fig. 4). While the purified TCP-1 complex can support protein refolding (17–19), it does not appear to be the major component in reticulocyte lysate responsible for the present renaturation activity.

In vitro, both hsp70 and hsp90 have been demonstrated to chaperone protein folding (7, 30). If either of these hspS mediates the renaturation of luciferase, luciferase renaturation should be proportional to the concentration of one or both of these hspS in the reticulocyte lysate. We measured the luciferase renaturation activity in four lysate preparations whose concentrations of hsp90 and hsp70 varied by approximately 4- and 6-fold, respectively (Fig. 5). A statistically significant correlation was found between the level of luciferase renaturation and the levels of both hsp70 (r = 0.953, p < 0.02) and hsp90 (r = 0.951, p < 0.02) present. These observations are consistent with a potential role for these proteins in the renaturation of luciferase in reticulocyte lysate.

Luciferase Renaturation in Immune Isolated Samples—Rabbit reticulocyte lysate has been shown to have relatively high concentrations of hsp90 and hsp70 (32–34). A third heat shock protein and potential molecular chaperone, p60, has also been shown to exist in reticulocyte lysate (51). p60 is not related to hsp60 but is homologous to the yeast heat shock protein STT1 (47, 51), and, while it aids in the protection of yeast from temperature stress, its functional significance is unknown (58). For more direct evidence that these proteins are involved in the chaperoning activity we observed, protein A-Sepharose-bound antibodies to hsp90 (D7a), hsp70 (BB70), and p60 (F5) were used to isolate these proteins and analyze their ability to renature luciferase. These three proteins have recently been shown to exist in a complex (51); therefore, immune isolations with any of these antibodies from reticulocyte lysate under low salt conditions results in protein complexes that contain the other two proteins as well (Fig. 6A). When these immune isolations were washed and suspended in 10 mm Tris-HCl, pH 7.5, 3 mm MgCl₂, 100 mm KCl, and 2 mm DTT and supplemented with an ATP-regenerating system, they support renaturation of denatured luciferase to restore 18–35% of the original activity (Fig. 6B). Recovery of luciferase activity in the absence of an ATP-regenerating system is less than 5%. If these same antibodies are used to immune isolate proteins under high salt conditions (0.4 M KCl), where complexes with other proteins are dissociated, they show less than 5% luciferase renaturation even in the presence of an ATP-regenerating system (data not shown).

Although the renaturation activity in immune isolated preparations is significant and ATP-dependent, this activity does not approach that of whole lysate. When concentrations higher than those shown in Fig. 6 are used, luciferase activities approach a maximum activity in the range of 25–35% that of lysate. This may be due to the steric constraints of immobilized proteins or because additional factors are lacking.

Luciferase Renaturation with Purified Proteins—Since the above experiments suggest that hsp90, hsp70, and p60 may be involved in luciferase renaturation, we pursued this further using purified proteins. hsp90, hsp70, and p60 were purified as described under "Experimental Procedures." In preliminary experiments, hsp90, hsp70, and p60, at concentrations similar to those found in reticulocyte lysate (50–150 μg/ml), did not individually support ATP-dependent reactivation of luciferase. However, when the three proteins were combined at the same concentrations, luciferase renaturation was greater than 15%. To determine whether all three of these proteins are required for luciferase renaturation, all combinations of the three proteins were tried (Fig. 7). The combination of hsp90 and hsp70 was the only one to support renaturation at a level similar to that found when all three proteins were present. The activity obtained was similar to that of immune isolated preparations but clearly below that of whole lysate.

In order to optimize luciferase renaturation and indicate the stoichiometry of hsp90 and hsp70 needed, the purified proteins were combined at various concentrations. Fig. 8 displays lucif-
Luciferase renaturation after the addition of different amounts of hsp90 to 75 μg/ml hsp70 and the effect that various amounts of hsp70 have on renaturation when added to 112 μg/ml hsp90. Taken together, these results suggest a mass ratio of close to 2:1 for hsp90 and hsp70, which is consistent with other reports that hsp90 acts as a dimer (44, 59–61). While excess hsp90 has little effect on the activity, the concentration curve for hsp70 reaches a maximum and then declines. The reason for this inhibitory effect of excess hsp70 is unknown, but inhibitory effects of hsp70 on protein folding have been noted by others (62). Luciferase renaturation activity with purified hsp90 and hsp70 is optimal at concentrations of 100–200 μg/ml (data not shown), which is near the concentrations of these proteins in reticulocyte lysate (32, 33, 63).

In additional experiments, the KCl dependency of the purified system was shown to closely resemble that of reticulocyte lysate. In the absence of KCl, activity was reduced to less than 50% of maximum. The optimum concentration of KCl was between 25 and 50 mM, and NaCl was unable to replace KCl in enhancing activity (data not shown).
**DISCUSSION**

In this report, we present an assay for the renaturation of mildly denatured luciferase. We have used this assay to examine the factors and conditions required for the renaturation of denatured luciferase and have identified ATP-dependent protein-chaperoning activity in reticulocyte lysate. The renaturation activity correlated well with hsp90 and hsp70 levels in various reticulocyte lysate preparations, and immune isolated samples from reticulocyte lysate that contain hsp70, hsp90, and p56 were also able to support limited ATP-dependent luciferase renaturation. These results prompted us to investigate whether purified samples of these proteins could support luciferase renaturation as well. Although none of these proteins alone are able to reactivate luciferase, hsp90 and hsp70 together are able to support luciferase renaturation.

Heat shock or addition of denatured BSA inhibited the luciferase renaturation activity present in the reticulocyte lysate, supporting the likelihood that acute heat shock “impairs” hsp function by inducing the accumulation of denatured protein, which binds and sequesters hsp.

Both the constitutively expressed forms of hsp70 and hsp90 are “insolubilized” in vivo during acute heat stress (64–67). hsp70 co-localizes with insolubilized cellular enzymes, which presumably represent aggregates of denatured proteins. After recovery from heat shock, recovery of endogenous and reporter enzyme activities (β-galactosidase and Firefly luciferase) occur upon concomitant resolubilization of the enzymes and hsp.

Our observations that luciferase renaturation activity correlates with the levels of hsp90 and hsp70 present in lysate and that hsp70 and hsp90 act in concert to catalyze luciferase renaturation suggest that the co-localization of these proteins during heat shock in vivo represents a functional interaction. In addition, the correlation between the rate of protein synthesis and luciferase renaturation activity (r = 0.952, p < 0.02) (data not shown) in four lysate preparations examined support the notion that the rate of cellular protein synthesis may be regulated in concert with the “chaperoning” capacity of the cell (32, 33).

In the present study, luciferase was denatured by mild heat treatment rather than by denaturants such as urea or guanidine hydrochloride. In addition, the enzyme was maintained in a buffer containing 1% Triton X-100, 10% glycerol, and 10 mg/ml BSA. These components stabilize the enzyme during storage, but they do not prevent its inactivation at 40 °C. However, they do prevent extensive aggregation of heat-treated luciferase and are required to maintain heat-treated luciferase in a state that allows renaturation by reticulocyte lysate or purified hsp70 and hsp90.

Furthermore, glycerol gradient sedimentation of luciferase has shown it to be mainly (>90%) in a monomeric form (~4 S) before and after heat-treatment (data not shown). Thus, the enzyme is likely to be only partially unfolded under our experimental conditions. We chose this procedure in an attempt to minimize the number of chaperoning activities that would be required for renaturation. A recent report by Nimmesgern and Hartl (37) described the refolding of Firefly luciferase in rabbit reticulocyte lysate after the enzyme had been fully denatured in 6 M guanidine hydrochloride. Whether this is a more demanding renaturation process than the one described here is unknown, since the chaperoning proteins involved have not yet been identified.

The activity observed with immune isolated proteins and purified hsp90 and hsp70 is less than that of whole lysate; therefore, some attempts were made to supplement the system with additional proteins (data not shown). DnaJ and p59 are two proteins that may play a role in protein folding and maturation. p59 is an immunophilin that binds the immunosuppres-
for the two bacterial heat shock proteins, DnaK, DnaJ, and GrpE. The present study indicates a functional unit of hsp70 and hsp90 that may participate in the folding of proteins or in the correction of folding errors. While the purified system described here is probably incomplete, it offers approaches for identifying additional factors and for characterizing the functional significance of individual protein components.

Acknowledgments—We thank Bridget A. Stensgard for technical assistance and Ronald K. Corbisier for assistance in the purification of reticulocyte lysate that has not been fully identified (11–13). Thus, data are accumulating that suggest that molecular chaperones act cooperatively or in larger functional units (6, 7, 34). In a more recent example, Schroder et al. (83) have used a denatured luciferase assay similar to the one described here to measure chaperoning activity of bacterial proteins. They found that lucerferase renaturation required the three heat shock proteins DnaK, DnaJ, and GrpE. The present study indicates a functional unit of hsp70 and hsp90 that may participate in the unfolding of proteins or in the correction of folding errors. While the purified system described here is probably incomplete, it offers approaches for identifying additional factors and for characterizing the functional significance of individual protein components.

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