Purification and Characterization of a 66-kDa Protein from Rabbit Reticulocyte Lysate Which Promotes the Recycling of Hsp 70*

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We have purified to apparent homogeneity a 66-kDa protein from rabbit reticulocyte lysate which is associated with hsp 70. Our characterization of this 66-kDa protein demonstrates that its physiological role is to promote the recycling of hsp 70 by catalyzing the dissociation of hsp 70-bound ADP in exchange for ATP. We have therefore termed the 66-kDa protein RF-hsp 70, a recycling factor for hsp 70. RF-hsp 70 promotes stoichiometric binding of ATP to hsp 70, and it increases about 5-fold the rate of dissociation of hsp 70ADP in the presence of ATP. This process represents adenine nucleotide exchange, since dissociation of ADP does not occur unless ATP is added; dATP, GTP, and ITP cannot substitute for ATP. The mechanism of action of RF-hsp 70 is to lower the $k_z$ of hsp 70 for ATP about 6-7-fold to a value that is close to the $k_z$ of hsp 70 for ADP. RF-hsp 70 also stimulates the ATPase activity of hsp 70, including the dissociation of bound ADP. RF-hsp 70 stimulates the ATPase activity of hsp 70 about 3-4-fold. The action of RF-hsp 70 is also partially species-specific since it is most effective with rabbit reticulocyte hsp 70, less effective with bovine brain hsp 70, and ineffective with human hsp 70 and ineffective with bovine brain hsp 70, and ineffective with chicken hsp 70. RF-hsp 70 acts catalytically to recycle hsp 70, since, at 0.2 times the molar concentration of hsp 70, RF-hsp 70 increases the rate of renaturation of luciferase by hsp 70 about 3-4-fold. The action of RF-hsp 70 is also partially species-specific since it is most effective with rabbit reticulocyte hsp 70, less effective with bovine brain hsp 70, and ineffective with human hsp 70, and ineffective with broad bean hsp 70.

We recently reported (1) the purification of rabbit reticulocyte hsp 70,1 termed hsp 70(R), a protein we previously had termed the supernatant factor (2,3). We demonstrated (1) that hsp 70(R) participates in the translational control of protein synthesis by hsm in reticulocyte lysate, as reported earlier by Mats and co-workers (4-6), by binding to and regulating the activation of the hsm-controlled, eIF-2 kinase (HCR). In that report (1), we noted that a 66-kDa protein, now termed RF-hsp 70, copurifies with hsp 70 until the final purification step. We chose to purify and characterize RF-hsp 70 because of its close association with hsp 70. This characterization indicates that RF-hsp 70 catalyzes the recycling of hsp 70 by promoting the dissociation of ADP bound to hsp 70 and its replacement by ATP. Previous studies have demonstrated that hsp 70 binds to nascent polypeptides or proteins that are unfolded or incorrectly folded, promotes proper folding, and then dissociates as hsp 70ADP in a reaction that involves hydrolysis of hsp 70ATP by the hsp 70 ATPase (7-13). Consequently, recycling of hsp 70 must involve dissociation of bound ADP and replacement by ATP. This process may be rate-limiting since the $k_z$ of bovine brain hsp 70 (hsp 70(B)) for ATP is 6-fold lower than that for ATP (14). We will demonstrate that RF-hsp 70 reduces the $k_z$ of ATP for hsp 70(R) or hsp 70(B) to a value that is close to the respective $k_z$ values for ATP and ADP and hence enhances the recycling of these hsp 70s. The recycling role of RF-hsp 70 may be similar to that envisioned for activities termed DnaJ and GrpE in bacteria (15) and YDJ 1 in yeast (16).

EXPERIMENTAL PROCEDURES

Purification of Protein Components—The purification of hsp 70(R) was described previously (1). The purification of RF-hsp 70 is identical to that of hsp 70(R) through the chromatography on phosphocellulose, step 4 (1). Chromatography of 120 mg of hsp 70 on a 1.0 x 4.5-cm ATP-agarose column (Sigma), as described (1), leads to the selective adsorption of hsp 70, a 72-kDa band, and a 66-kDa band, RF-hsp 70(A) (Fig. 1, lanes 2 and 3). After washing the column with 70 ml of buffer A (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 3 mM MgCl$_2$, 1 mM dithiothreitol, and 0.1 mM EDTA), the column was eluted with 30 ml of buffer A, containing 300 mM KCl, collected in 2 ml, and then eluted with 24 ml of buffer A containing 3 mM ATP. The 300 mM KCl eluate contains the 66-kDa band of RF-hsp 70, which comprises about 70% of the total protein, and virtually no hsp 70(B) (Fig. 1, lanes 4 and 5). In contrast, hsp 70(R) is eluted selectively with 3 mM ATP (Fig. 1, lane 8) because of the strong ATP binding site characteristic of the hsp 70 family of proteins (17). The adsorption of RF-hsp 70 (66-kDa protein) to ATP-agarose appears to be due to the association of RF-hsp 70 with hsp 70(R) and not binding to ATP-agarose, since ATP-agarose-purified RF-hsp 70, which is almost completely free of hsp 70(R), fails to bind when it is recombinantly adsorbed on ATP-agarose (data not shown).

The RF-hsp 70 in the 300 mM KCl eluate from ATP-agarose (step 5) was concentrated by ultrafiltration and then purified to apparent homogeneity by either sucrose density gradient centrifugation as described below or chromatography on Mono Q (Pharmacia Biotech Inc.). In the latter case, 1.5 mg of step 5, RF-hsp 70 was applied to a 0.5 x 5.0-cm (1.0 ml) Mono Q column, equilibrated in 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.10 mM EDTA (buffer B). After washing with 3.0 ml of buffer B, the column was eluted with a linear gradient (20 ml) of buffer B containing 450 mM KCl at a flow rate of 1.0 ml/min. RF-hsp 70 (66-kDa band), purified to apparent homogeneity (Fig. 1, lane 6), eluted at 200 mM KCl, received a final concentration of 1.0 mM dithiothreitol, and was stored in liquid nitrogen at a final concentration of 0.5 mg/ml. The initial purification of rabbit reticulocyte hsp 70 was the same as that for hsp 70(R) through the ammonium sulfate step (2,3). The hsp 90

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1 The abbreviations used are: hsp 70, a member of the 70-kDa heat shock protein family; hsp 70(R), rabbit reticulocyte hsp 70; eIF, eukaryotic initiation factor; eIF-2, the e or smallest subunit of eIF-2; HCR, the hemin-controlled translational repressor or eIF-2 kinase; RF-hsp 70, a 66-kDa rabbit reticulocyte protein that recycles hsp 70; hsp 70(B), bovine brain hsp 70; hsp 70, a member of the 70-kDa heat shock protein family; hsp 70(H), human hsp 70 (recombinant protein); hsp 70(bn), broad bean hsp 70; polypeptide A, amino acid residues 41-54 of eIF-2 alpha, the smallest subunit of eIF-2; TCP-1, the large hetero-oligomeric t-complex protein 1; hsp70(B), a member of the 70-kDa heat shock protein family; hsp90, a member of the 90-kDa heat shock protein family; hsp90(B), bovine brain hsp 90; the site phosphorylated by HCR; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]lysine; TCP-1, the large hetero-oligomeric t-complex protein 1 (also termed TCP-1).
chymotrypsin digestion, is identical to the carboxyl-terminal 35 residues of the constitutive form of rat hsp 70 (19, 20), confirming that the hsp 70(R) we have purified is the constitutively expressed form of the protein. Broad bean hsp 70, termed hsp 70(bn), was generously provided by Matthew A. Harney of The National University of Ireland. It shows a single broad band with an estimated size of 68 kDa on gel analysis (Fig. 1, lane 11).

Preparation of Polyepitope A—Amino acid residues 41–54 of eIF-2α, representing the sequence IEGRILLSLRRRR and comprising the phosphorylation site of HCR (21), was synthesized on a Applied Biosystems 430a peptide synthesizer and kindly provided by Stephen Meredith at The University of Chicago. This product, termed polyepitope A, was purified by reverse-phase chromatography on an Aqueapore RP-300 column (4.6 × 250 mm; Brownlee Laboratories) using a 0–50% acetonitrile gradient in 0.05% (v/v) trifluoroacetic acid in water at a flow rate of 1.0 ml/min over a 60-min period.

Gel Electrophoresis and Immunoblotting—Electrophoresis of protein samples on 7% polyacrylamide-SDS slab gels (22) followed by either silver staining (23) or electrophoretic transfer to nitrocellulose sheets (at 50 V for 7 h in a Hoefer Transphor) and then immunoblotting (24) has been described previously.

Sucrose Gradient Centrifugation—Protein samples were layered over 4.4-ml 5–20% (w/v) linear sucrose gradients and centrifuged in a Beckman L5-65 ultracentrifuge for 17.5 h at 54,000 rpm (300,000 × gmax) and 2°C in the SW 60-Ti rotor. Gradients were pumped out by upward displacement and divided into 35 0.13-ml fractions. Gradient buffer into which the samples were loaded contained 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol, and 0.1 mM EDTA. Gradient buffer used in the purification of RF-hsp 70 was similar, but MgCl2 was omitted.

Renaturation of Luciferase—The ability of hsp 70, hsp 90, and RF-hsp 70 to promote the renaturation of luciferase was assessed as described by Schumacher et al. (18). Firefly luciferase (Sigma) was diluted in 25 mM Tricine, pH 7.8, 10 mM MgCl2, 1 mM dithiothreitol, 0.1 mM EDTA, 10% (v/v) glycerol, and 10 mg/ml bovine serum albumin (buffer C) to a final concentration of 0.065 mM. Duplicate aliquots were removed for determination of the initial activity, and the remainder was heated at 40°C for 20 min, reducing luciferase activity to an average of 5–8% of its initial activity. When samples were renatured for a single period of time, renaturation reactions, in duplicate, contained 2.7 μl of heated luciferase, 0.50 mM ATP, 15 mM creatine phosphate, 45 units/ml creatine phosphokinase, and the protein additions indicated in the figure and table legends. Results are expressed as the percentage of the initial activity of unheated luciferase.

Materials—Reduced and carboxymethylated γ-lactalbumin (RCM-αLB) and reagents for the renaturation and assay of firefly luciferase were all from Sigma, except that dithiothreitol was purchased from CalBiochem. [α-32P]ATP was obtained from ICN. Reagents for gel electrophoresis were bought from Bio-Rad. Electrophoresis, gel transfer, and gel drying equipment were from Hoefer, and ultrafiltration supplies were from Amicon.

RESULTS

Purification of RF-hsp 70—The purification of RF-hsp 70 from rabbit reticulocyte lysate, as described under “Experimental Procedures,” is shown in Table I. Activity is based upon stimulation of the renaturation of luciferase by hsp 70(R) and is

M. Gross, unpublished observation.
characterized in detail later (see Figs. 8 and 9 and Table IV).

Based upon this activity, RF-hsp 70 was purified approximately 840-fold with a yield of about 2%. One explanation for the relatively low yield of RF-hsp 70 in the purification is that appreciable 66-kDa protein and activity separate into side fractions at steps 2, 3, and 5. In addition, using immunoblot analysis with polyclonal antibody raised to hsp 70(R), which reacts only with the 72-kDa band of hsp 70(R) and the 66-kDa band of RF-hsp 70 (1), we estimate that the concentration of the 66- and 72-kDa proteins in rabbit reticulocyte lysate is about 0.09 and 0.24 mg/ml, respectively (data not shown). This indicates that the recovery of the 66-kDa protein is approximately 3.6%, and its purification is about 1,600-fold. This suggests that one or more proteins in the lysate, separate from the 66-kDa protein (RF-hsp 70), also promote the renaturation of luciferase in the presence of hsp 70(R) and could account for up to 50% of such activity. One possibility is hsp 90, which has been shown previously to have such activity (Ref. 18 and see Fig. 8) and is separated from the 66-kDa protein at the DEAE-cellulose step (see Fig. 1). Another possibility that has been suggested (27) is the large hetero-hologenic ring complex TCP-1. Alternatively, RF-hsp 70 may undergo partial inactivation (up to 50%) in the first several steps of its purification. In either case, we have observed that RF-hsp 70 activity, measured as indicated in Table I, copurifies with the 66-kDa protein from steps 3–6 in the purification (see Fig. 1).

Effect of RF-hsp 70 on the Binding of ATP to Hsp 70 and on Adenine Nucleotide Exchange—Our observation that RF-hsp 70 and hsp 70(R) appear to be associated with each other during their purification prompted us to test whether this association is physiologically significant. Therefore, we examined the ability of hsp 70(R) to bind $[^{32}P]ATP$ in the absence or presence of an equimolar concentration of RF-hsp 70. The results (Fig. 2A) demonstrate increasing ATP binding to hsp 70(R) with increasing ATP concentration, reaching a maximum of 1.0 mol/mol in the presence of RF-hsp 70 and about 0.6 mol/mol in the absence of RF-hsp 70 at ATP concentrations of 8 $\mu$M and greater. Half-maximal binding to hsp 70(R) occurs at about 1.0 and 1.6 $\mu$M ATP in the presence and absence of RF-hsp 70, respectively. For comparison, $[^{32}P]ATP$ binding to hsp 70(B) requires about 100 $\mu$M ATP for maximal binding (and about 30 $\mu$M ATP for half-maximal binding), that is 1.0 mol/mol in the presence of RF-hsp 70 and 0.5 mol/mol in its absence (Fig. 2B). In contrast to hsp 70(R) and hsp 70(B), RF-hsp 70 shows no significant ATP binding at concentrations of ATP up to 105 $\mu$M (data not shown), indicating that the binding site is not on the ATPase domain.

This explanation was tested by determining the effect of RF-hsp 70 on the rate of dissociation of radioactivity from hsp 70(R), prelabeled with $[^{32}P]ATP$, upon subsequent incubation in the presence of excess unlabeled ATP. As shown in Table II, experiment A, there is a slow rate of dissociation of radiolabel from hsp 70(R) in the presence of unlabeled ATP which is increased approximately 6-fold by an equimolar concentration of RF-hsp 70. Even one-third as much RF-hsp 70 stimulated the rate of dissociation 2-fold, suggesting that RF-hsp 70 action on hsp 70(R) is catalytic rather than stoichiometric.

TABLE I

| Step                  | ml  | units/ml | mg/ml | units/mg | Purification | Yield |
|-----------------------|-----|----------|-------|----------|--------------|-------|
| 1. Lysate             | 4,000 | 215      | 145   | 1.5      | 1.0          | 100   |
| 2. Ammonium sulfate   | 320  | 750      | 50    | 15       | 10           | 28    |
| 3. DEAE-cellulose     | 50   | 1,880    | 11    | 170      | 113          | 11    |
| 4. Phosphocellulose   | 56   | 1,440    | 8.0   | 180      | 120          | 9.3   |
| 5. ATP-agarose        | 8.1  | 2,560    | 2.5   | 1,020    | 680          | 2.4   |
| 6. Gradient centrifugation or Mono Q | 26 | 630     | 0.50  | 1,260    | 840          | 1.9   |

**FIG. 2. Potentiation by RF-hsp 70 of ATP binding to hsp 70(R) and hsp 70(B).** Binding reactions were in 5 ml Tris-HCl, pH 7.3, 85 mM KCl, 5 mM NaCl, 2.5 mM MgCl$_2$, 0.5 mM dithiothreitol, and 0.05 mM EDTA and contained 15 mM creatine phosphate and 45 units/ml creatine phosphokinase (to ensure added ATP would be fully in triphosphate form) and 0.4 $\mu$g of hsp 70(R) (panel A) or hsp 70(B) (panel B) in a final volume of 12 ml. After preincubation at 34°C for 0.5 min in the presence (●) or absence (○) of 0.4 $\mu$g of RF-hsp 70, samples were incubated for 1.0 min more with the indicated concentrations of $[^{32}P]ATP$ (2.10$^6$ cpm/pmol), and ATP bound to hsp 70 was determined by Millipore filtration. The results are an average of two separate determinations. The binding shown has been corrected by subtracting the binding obtained in blank reactions containing RF-hsp 70 alone or neither hsp 70 nor RF-hsp 70.
Effect of RF-hsp 70 on the dissociation (exchange) of ADP bound to hsp 70

Samples, containing 0.5 mg/ml hsp 70, 2.5 mM MgCl₂, and 0.16 mM \([\alpha^{-32}P]ATP\) (12 \(\cdot\) \(10^4\) cpm/pmol), were preincubated for 8 min at 34°C. Reactions were constituted as described in the legend to Fig. 2, but creatine phosphate was omitted from those samples receiving added ADP. Samples in experiment A contained 0.7 \(\mu\)g of hsp 70(R), ATP (0.50 mM), and the indicated amount of RF-hsp 70. Samples in experiment B contained 0.7 \(\mu\)g of hsp 70(R), 0.7 \(\mu\)g of RF-hsp 70 (when added), and the indicated nucleotide (0.50 mM). Samples in experiment C contained 0.7 \(\mu\)g of hsp 70, ATP (0.50 mM), and 0.7 \(\mu\)g of RF-hsp 70 (where added). The final volume was 23 \(\mu\)l, and incubation was at 34°C. The rate of dissociation (nmol/min/ml) of radioactivity, prebound to hsp 70, was determined by Millipore filtration of 4.0-\(\mu\)l aliquots (five total) removed from each sample at 20-s intervals. Thin layer chromatography on polyethyleneimine-cellulose of 5% (w/v) trichloroacetic acid extracts of Millipore filters indicated that the radioactivity bound to hsp 70(R) after the 8-min preincubation was 76% ADP and 24% ATP and that this proportion was not altered appreciably by 3.0 min of further incubation in the presence of creatine kinase and creatine phosphate. The results are an average of two separate determinations.

| Experiment A | Dissociation | Addition | Dissociation | Experiment B | Dissociation | Addition | Dissociation | Experiment C | Dissociation |
|--------------|--------------|----------|--------------|--------------|--------------|----------|--------------|--------------|--------------|
| RF-hsp 70    | 0.035        | ATP      | 0.023        | hsp 70(R)    | 0.028        | RF-hsp 70+ATP | 0.156        | RF-hsp 70    | 0.154        |
| 0.6 \(\mu\)g | 0.199        |           |              | hsp 70(R)    | 0.028        | RF-hsp 70+ATP | 0.062        | RF-hsp 70    | 0.154        |
| 0.4 \(\mu\)g | 0.141        | ADP      | 0.085        | hsp 70(B)    | 0.018        | RF-hsp 70+GTP | 0            | RF-hsp 70    | 0.064        |
| 0.2 \(\mu\)g | 0.101        |           |              | hsp 70(B)    | 0.018        | RF-hsp 70+ITP | 0            | RF-hsp 70    | 0.064        |
|              |              | RF-hsp 70+ dATP | 0            |              |              | RF-hsp 70    | 0.064        |              |              |

**FIG. 3. Effect of RF-hsp 70 on the rate of binding of ATP to hsp 70(R) and hsp 70(B), containing prebound adenine nucleotide.**

Samples, containing 0.5 mg/ml hsp 70, 2.5 mM MgCl₂, and 0.16 mM ATP, were preincubated for 8 min at 34°C. Reactions constituted as described in the legend to Fig. 2, and containing 0.7 \(\mu\)g of hsp 70(R) (circles) or hsp 70(B) (triangles), were reincubated at 34°C in the presence (closed symbols) or absence (open symbols) of 0.7 \(\mu\)g of RF-hsp 70 and with added \([\alpha^{-32}P]ATP\) (to give a final ATP concentration of 20 \(\mu\)M and specific activity of 7 \(\cdot\) \(10^4\) cpm/pmol) in a final volume of 23 \(\mu\)l. Aliquots (5.0 \(\mu\)l) were removed at the indicated times, and the binding of \([\alpha^{-32}P]ATP\) was determined by Millipore filtration. Binding reactions were in duplicate, and the binding shown has been corrected for binding in blank reactions as described in the legend to Fig. 2.

Rebinding of ATP shown in Fig. 3, demonstrating that a majority of the dissociated nucleotide is ADP. The dissociation of ADP from hsp 70(R) promoted by RF-hsp 70 is strictly dependent upon the addition of ATP, since no dissociation occurs in the presence of GTP, ITP, or dATP (Table II, experiment B) or in the presence of added nucleotide (data not shown). This indicates that RF-hsp 70 catalyzes the dissociation of ADP from hsp 70(R) by promoting the exchange of bound ADP for ATP. Dissociation of radiolabel from hsp 70(R) in the presence of ADP (presumably reflecting exchange of \([\alpha^{-32}P]ADP\) with unlabeled ADP) is almost 4-fold faster than in the presence of ATP (Table II, experiment B) and is not stimulated by RF-hsp 70. This suggests that RF-hsp 70 promotes only the physiologically relevant reaction where ADP bound to hsp 70(R) is replaced with ATP. Results identical to those shown in Table II, experiment B, were obtained using hsp 70(R), complexed primarily with \([\alpha^{-32}P]ADP\), which had been isolated free of unbound, radioactive adenine nucleotide by chromatography on Sephadex G-25 following preincubation with \([\alpha^{-32}P]ATP\) as described in the legend to Table II (data not shown). The effect of RF-hsp 70 is not limited to hsp 70(R), since RF-hsp 70 also stimulates the dissociation of ADP bound to hsp 70(B) in the presence of ATP, although its effect on hsp 70(B) is less than that on hsp 70(R) (Table II, experiment C).

To verify that the dissociation of adenine nucleotide (primarily ADP) from hsp 70 in Table II is associated with binding of ATP (i.e. adenine nucleotide exchange), hsp 70(R) and hsp 70(B) were preincubated with ATP and MgCl₂ to produce maximal binding of unlabeled nucleotide. We then determined the effect of RF-hsp 70 on the rate of binding of \([\alpha^{-32}P]ATP\) to each hsp 70, which would be dependent upon dissociation of bound nucleotide. Binding of labeled ATP to each hsp 70 in the absence of RF-hsp 70 is relatively slow (Fig. 3), presumably because of the slow rate of dissociation of bound adenine nucleotide shown in Table II. In contrast, initial \([\alpha^{-32}P]ATP\) binding to each hsp 70 in the presence of RF-hsp 70 is about six times faster (Fig. 3), similar to the increased rate of dissociation of bound nucleotide induced by RF-hsp 70 (Table II). This result confirms that the dissociation of adenine nucleotide from hsp 70 promoted by RF-hsp 70 represents adenine nucleotide exchange. The extent of binding of \([\alpha^{-32}P]ATP\) to hsp 70(R) is approximately 1.75 times that to hsp 70(B) (Fig. 3), presumably because the concentration of ATP in the binding reaction, 20 \(\mu\)M, permits maximal binding to hsp 70(R) but not hsp 70(B) (Fig. 2).

Effect of RF-hsp 70 on the ATPase Activity of Hsp 70—If RF-hsp 70 promotes the recycling of hsp 70 by increasing the rate of dissociation of ADP and binding of ATP, as indicated by the results in Table II and Fig. 3, one would expect the rate of hydrolysis of ATP to ADP by hsp 70 to be increased similarly by RF-hsp 70. This is confirmed experimentally, since the ATPase activity of hsp 70(R) (0.035 nmol/min/ml) is increased 6-fold (to 0.21 nmol/min/ml) when incubated with an equimolar (and saturating) concentration of RF-hsp 70 (Fig. 4). RF-hsp 70 by
itself has no significant ATPase activity. RF-hsp 70 appears to act catalytically on hsp 70(R), since one-fourth as much RF-hsp 70 still has close to a saturating effect on its ATPase activity. This effect of RF-hsp 70 is partially specific to hsp 70(R), since RF-hsp 70 has progressively less ability to stimulate the ATPase activity of the native hsp 70 (Fig. 6) to convert both hsp70(R) and hsp70(B) almost entirely to a product with an estimated molecular mass of 42 kDa (as judged by SDS-polyacrylamide gel electrophoresis), which appears to be slightly smaller than the 44-kDa component characterized by Chappell et al. (29). As noted by Chappell et al. (29), the ATPase activity of the 42-kDa component derived from hsp70(B) is considerably greater (about 8-fold more) than that of the native hsp 70 (Fig. 6B). Conversion to the 42-kDa component also increases the ATPase activity of hsp70(R) (Fig. 6A), but not to the same degree. In addition, whereas the ATPase activity of hsp70(B) (Fig. 6B) is stimulated (about 8-fold and 5-fold, respectively) by polypeptide A, the ATPase activity of the 42-kDa component derived...
from each hsp 70 is not stimulated by polypeptide A. This result is consistent with the findings of Chappell et al. (29) and supports the belief that a carboxy-terminal domain in hsp 70 is required for its interaction with unfolded protein or polypeptide. In contrast, RF-hsp 70 increases the ATPase activity of each 42-kDa component and the activity of the corresponding native hsp 70 by similar digestion with chymotrypsin. The remainder of the procedure was as described in the legend to Fig. 4. The results are an average of two separate experiments. SDS-gel electrophoresis and silver staining of aliquots of the chymotrypsin digest demonstrated that almost all of the initial hsp 70 had been removed and that the predominant product was a 42-kDa component that was quantified by laser densitometry.

Mechanism of Action of RF-hsp 70—To examine the mechanism by which RF-hsp 70 increases the rate of dissociation of hsp 70-ADP and the binding of ATP (Table I and Fig. 3), resulting in the stimulation of hsp 70 ATPase activity (Figs. 4–6), we measured the binding of [$\gamma$-32P]ATP and ADP to hsp 70(R) and hsp 70(B) and analyzed the results by Scatchard plot (30) with the findings summarized in Table III. They indicate that RF-hsp 70 has no appreciable effect on the $K_D$ for ADP of either hsp 70(R) or hsp 70(B). Instead, RF-hsp 70 reduces the $K_D$ for ATP of hsp 70(R) from 1.2 to 0.22 $\mu$M and that of hsp 70(B) from 9.9 to 1.4 $\mu$M, values that are close to the $K_D$ for ADP of hsp 70(R) (0.15 $\mu$M) and hsp 70(B) (1.1 $\mu$M). These results suggest that RF-hsp 70 promotes the dissociation of hsp 70-bound ADP and the binding of ATP by increasing the affinity of hsp 70 for ATP approximately 6-fold to a value that is close to the affinity of hsp 70 for ADP. Our values for the $K_D$ of hsp 70(B) for ATP (9.9 $\mu$M) and ADP (1.7 $\mu$M), which were determined by Millipore filtration (Table III), are in close agreement with the values (9.5 and 1.6 $\mu$M, respectively) reported by Palleros et al. (14) which were determined by equilibrium dialysis. The finding that RF-hsp 70 does not alter the $K_D$ of hsp 70(R) for ADP correlates with our observation (Table II, experiment B) that RF-hsp 70 has no effect on the rate of exchange of radioactive for unlabeled ADP bound to hsp 70(R). This reinforces the belief that RF-hsp 70 promotes only the physiologically relevant exchange reaction where hsp 70-bound ADP is replaced by ATP.

To determine whether the action of RF-hsp 70 on hsp 70(R) involves stable association of these two proteins, RF-hsp 70 and hsp 70(R) were preincubated either separately or together and then fractionated on sucrose density gradients. Aliquots of gradient fractions were analyzed for RF-hsp 70 (66-kDa band) and hsp 70(R) (72-kDa band) by SDS-polyacrylamide gel electrophoresis, silver staining, and laser densitometry. The results of Fig. 7 demonstrate that RF-hsp 70 alone sediments entirely as a single component with a peak at fraction 16, migrating somewhat slower than rabbit hemoglobin (peak at fractions 18–19), and hsp 70(R) alone shows a major peak migrating at fraction 19 or slightly faster than rabbit hemoglobin. These findings are in reasonable agreement with the estimated molecular sizes of RF-hsp 70 and hsp 70(R) from SDS-polyacrylamide gel electrophoresis, and they indicate therefore that RF-hsp 70 is a single polypeptide chain. Gradient analysis of hsp 70(R) alone also shows a faster sedimenting component with a peak at fraction 26, which likely represents a homodimer, as we noted previously (1) and as has been observed with other hsp 70 species (14, 20). Analysis of RF-hsp 70 preincubated with hsp 70(R) shows the same peaks seen with each protein alone as well as two additional peaks. One, sedimenting with a peak at fraction 24 and containing approximately equal amounts of RF-hsp 70 and hsp 70(R) stainable protein, is presumably a RF-hsp 70-hsp 70(R) complex. The other, sedimenting at fractions 31–32 and containing about 1.5 times more hsp 70(R) than RF-hsp 70 stainable protein, may represent a RF-hsp 70-hsp 70(R)-hsp 70(R) complex. These results demonstrate that RF-hsp 70 and hsp 70(R) stably associate with each other. Consistent with a catalytic effect of RF-hsp...
70 on hsp 70(R) recycling, preincubation of RF-hsp 70 and hsp 70(R) in the presence of ATP eliminates most of the complex formation between these two proteins seen in Fig. 7 (data not shown).

Effect of RF-hsp 70 on the Renaturation of Luciferase by Hsp 70—To demonstrate that RF-hsp 70 catalyzes the recycling of hsp 70 in a functional assay, we tested its effect on the renaturation of denatured firefly luciferase by hsp 70, as described recently (18, 27). The data in Fig. 8 show the rate of renaturation of heat-denatured luciferase in an isolated reaction containing an ATP-regenerating system. Pretreatment of luciferase for 20 min at 40°C reduced its activity to 5% of the initial activity (zero time in Fig. 8), and reincubation at 32°C with only the ATP-regenerating system resulted in no renaturation over a 90-min period. Reincubation with 0.070 mg/ml hsp 70(R), a concentration that is near saturating (see also Fig. 9 over a 90-min period. Reincubation with 0.070 mg/ml hsp 70 alone has almost no effect (data not shown). These effects are similar to those observed by Schumacher et al. (18). In contrast, reincubation with 0.070 mg/ml hsp 70(R) and an equimolar concentration of RF-hsp 70 increases the initial rate of reactivation of luciferase more than 7-fold relative to hsp 70(R) alone, leading to the restoration of 42% of the initial luciferase activity after 90 min (Fig. 8). The same concentration of RF-hsp 70 alone has almost no reactivation effect (Fig. 8), and RF-hsp 70 does not promote renaturation in the presence of hsp 90 (data not shown). Consistent with a catalytic effect, 0.012 mg/ml RF-hsp 70 increases the rate of renaturation of luciferase by 0.070 mg/ml hsp 70(R) about three times faster than hsp 70(R) alone, matching the effect of limiting (0.035 mg/ml) hsp 70(R) plus an equimolar concentration of RF-hsp 70 (Fig. 8). Although hsp 90 increases the rate of luciferase renaturation when incubated with hsp 70(R) alone, it does not increase the renaturation produced by hsp 70(R) plus hsp 90 (Fig. 8), and RF-hsp 70 does not promote renaturation of luciferase by 0.070 mg/ml hsp 70(R) about three times faster than hsp 70(R) alone, matching the effect of limiting (0.035 mg/ml) hsp 70(R) plus an equimolar concentration of RF-hsp 70 (Fig. 8).

Fig. 7. Formation of a complex between hsp 70(R) and RF-hsp 70. Samples, containing 30 μg of hsp 70(R) (●), 30 μg of RF-hsp 70 (▲), or 30 μg of hsp 70(R) and 30 μg of RF-hsp 70 (○, △), were incubated in 30 μl of gradient buffer for 5 min at 34°C. Each then received 60 μl of (0°C) gradient buffer and was fractionated by sucrose gradient centrifugation as described under “Experimental Procedures.” Aliquots (15 μl) of gradient fractions were analyzed by SDS-polyacrylamide gel electrophoresis and silver staining. The staining intensity of the 72-kDa band (circles) of hsp 70(R) and the 66-kDa band (triangles) of RF-hsp 70 was quantitated by laser densitometry using a Pharmacia Ultrascan XL. The arrow at the top indicates the sedimentation position of rabbit hemoglobin that was centrifuged in a parallel gradient, and the direction of sedimentation was from left to right.

Fig. 8. Effect of hsp 70(R), RF-hsp 70, and hsp 90 on the rate of renaturation of luciferase. Duplicate samples of heat-denatured luciferase were incubated under renaturation conditions at 32°C in the presence of: no further addition (●), 0.063 mg/ml RF-hsp 70(○), 0.035 mg/ml hsp 70(R) and 0.032 mg/ml RF-hsp 70(△), 0.12 mg/ml hsp 70(R) and 0.11 mg/ml RF-hsp 70(□), or 0.070 mg/ml hsp 70(R) and no further addition (●) or with 0.15 mg/ml hsp 90(●), 0.063 mg/ml RF-hsp 70(○), 0.063 mg/ml RF-hsp 70 and 0.15 mg/ml hsp 90(△), or 0.012 mg/ml RF-hsp 70(□). At the indicated times, 25-μl aliquots were removed for the determination of luciferase activity as indicated under “Experimental Procedures.” The results are an average of two separate experiments.

Fig. 9. Effect of varied concentrations of hsp 70(R) and RF-hsp 70 on the renaturation of luciferase. Duplicate samples of heat-denatured luciferase were incubated for 60 min under renaturation conditions in the presence of 0.035 (triangles), 0.070 (circles), or 0.12 (squares) mg/ml hsp 70(R) and the indicated molar ratio of RF-hsp 70 to hsp 70(R) (panel A) or in the presence of the indicated concentrations of hsp 70(R) and in the presence (●) or absence (○) of RF-hsp 70 at 0.50 times the molar concentration of the added hsp 70(R) (panel B). Luciferase activity was then determined as indicated under “Experimental Procedures.” Aliquots of heat-denatured luciferase which were not incubated or incubated without hsp 70(R) or RF-hsp 70 (panel A) had 7.5% of the initial luciferase activity. The results in panel A are a composite of five experiments, and those in panel B are an average of two experiments.

RF-hsp 70 (Fig. 8). A saturating renaturation effect (see also Fig. 9A) is produced by reincubation with 0.12 mg/ml hsp 70(R) and an equimolar concentration of RF-hsp 70 (Fig. 8). This increases the initial rate of renaturation of luciferase 10-fold.
faster than hsp 70(R) alone, leading to the restoration of 55% of the initial activity after 90 min.

Having established the kinetics of renaturation of luciferase with selected concentrations of hsp 70(R) and RF-hsp 70 (Fig. 8), we examined the effect of different concentrations and molar ratios of these two proteins on renaturation after a 60-min incubation at 32 °C (Fig. 9). Incubation with hsp 70(R) alone produces, at most, only a 2-fold restoration of luciferase activity with a saturating effect achieved at about 0.070 mg/ml (Fig. 9B). Incubation with hsp 70(R) plus a limiting concentration of RF-hsp 70 (added at one-half the molar concentration of hsp 70(R)) is much more effective, renaturing luciferase to 42% of its initial activity at 0.070 mg/ml hsp 70(R) and to a maximal 55–60% of initial activity at 0.12–0.18 mg/ml hsp 70(R). Interestingly, at concentrations below 0.070 mg/ml, hsp 70(R) (plus RF-hsp 70) is much less effective, producing, for example, only one-fourth the renaturation at one-half (0.035 mg/ml) the concentration (Fig. 9B). This sigmoidal concentration-dependence curve is independent of the RF-hsp 70 concentration, since hsp 70(R) produces almost four times the renaturation at 0.070, as at 0.035, mg/ml over a molar ratio of RF-hsp 70 to hsp 70(R) ranging from 0.20 to 1.6 (Fig. 9A). The data in Fig. 9A also demonstrate that, at limiting, near saturating, and saturating concentrations of hsp 70(R), a maximal effect of RF-hsp 70 is achieved when its concentration is equimolar to that of hsp 70(R). In addition, at saturating concentrations of hsp 70(R) and RF-hsp 70, luciferase is renatured to more than 70% of its initial activity. These data (Fig. 9A) also confirm the catalytic action of RF-hsp 70, since RF-hsp 70 increases the effect of 0.12 mg/ml hsp 70(R) 2.5- and 3.7-fold when added at 0.08 and 0.20 times, respectively, the molar concentration of hsp 70(R).

As noted by Schumacher et al. (18), the hsp 70(R) concentration of 0.070 mg/ml (or about 1 μM) which we have found to be near saturating in the renaturation reaction (Fig. 9) is almost 5 orders of magnitude greater than the luciferase concentration (0.013 nm). Consequently, we tested whether the same concentration of hsp 70(R) would be effective with much higher concentrations of denatured luciferase, since much higher concentrations of protein requiring chaperonin action are present in vivo. We found that in renaturation reactions (60 min at 32 °C) containing 1 μM hsp 70(R) and RF-hsp 70, heat-denatured luciferase, varying in concentration from 0.013 nm to 0.11 μM or 4 orders of magnitude, was similarly renatured from 7–10% to 58–71% of its initial activity (data not shown). This suggests that the RF-hsp 70-promoted chaperonin action of hsp 70(R) illustrated in Figs. 8 and 9 may apply under physiological conditions. It also should be noted that our estimate of the concentration of hsp 70(R) in rabbit reticulocyte lysate of 0.24 mg/ml is significantly above the concentration we have found to be saturating in an isolated luciferase renaturation assay.

We also compared the renaturation effect of different hsp 70s (at 0.070 mg/ml) in the absence or presence of an equimolar concentration of RF-hsp 70 (Table IV). Added alone, hsp 70(R), hsp 70(B), and hsp 70(H) each had about the same effect, increasing luciferase activity slightly less than 2-fold. In contrast, RF-hsp 70-stimulated renaturation is partly hsp 70-specific (Table IV), since RF-hsp 70 increases the renaturation by hsp 70(R) 7.4-fold, that by hsp 70(B) 4.0-fold, and that by hsp 70(H) only 2.8-fold. These results correlate with the differential stimulation by RF-hsp 70 of the ATPase activity of these hsp 70s (Fig. 4). They confirm the partial specificity of RF-hsp 70 action on different hsp 70s, and they strongly suggest that the promotion of adenine nucleotide exchange (resulting in enhanced ATPase activity) on hsp 70 by RF-hsp 70 underlies the enhanced chaperonin activity (renaturation of luciferase) of hsp 70 produced by RF-hsp 70.

| Added hsp 70 | % of Initial luciferase activity |
|-------------|---------------------------------|
| None        | 7.8                             |
| Hsp 70(R)   | 14.5                            |
| Hsp 70(B)   | 14.9                            |
| Hsp 70(H)   | 13.4                            |

**DISCUSSION**

Previous studies have demonstrated that the action of hsp 70 is mediated by binding to other proteins such as nascent polypeptides (7), immunoglobulin heavy chains, and unfolded, abnormally formed, or incorrectly folded proteins (8–13). This protein-protein interaction with hsp 70 is believed to result in the proper folding or refolding of newly or abnormally formed or denatured proteins. Dissociation of hsp 70 can then occur in the presence of ATP/Mg2+ and is associated with ATP hydrolysis by the hsp 70 ATPase (7–13). Recycling of hsp 70 is thought to involve release of hsp 70-ADP following ATP hydrolysis, rebinding to another polypeptide or protein, exchange of ATP for bound ADP, ATP hydrolysis, and dissociation. However, the precise sequence of events and whether the sequence of events varies or is the same for different hsp 70s and different protein-protein interactions are currently unclear (12). It is clear, however, that hsp 70 (bovine brain or rabbit reticulocyte) binds ADP 6–10 times more tightly than ATP (Table III and Ref. 14), suggesting that dissociation of ADP and binding of ATP may limit the rate of hsp 70 recycling.

We believe that the characteristics of RF-hsp 70 action on hsp 70, summarized below, indicate that ADP/ATP exchange is likely to be rate-limiting for hsp 70 recycling and that the physiological role of RF-hsp 70 is to promote the recycling of hsp 70. RF-hsp 70 promotes the dissociation of ADP from hsp 70-ADP, but only in the presence of ATP, by lowering the Kd of hsp 70 for ATP to a value that approaches the Kd of hsp 70 for ADP (Fig. 7); this effect of RF-hsp 70 is associated with the stimulation of hsp 70 ATPase activity and involves an interaction with the amino-terminal portion of hsp 70, in contrast to the effect of unfolded protein or polypeptide. RF-hsp 70 and hsp 70(R) are associated with each other in partially purified preparations from rabbit reticulocyte lysate, and the purified proteins re-form a complex when incubated together. Finally, RF-hsp 70 acts catalytically to stimulate up to 10-fold the ability of hsp 70 to reactivate heat-denatured luciferase, providing a functional demonstration of its recycling activity.

Our findings are consistent with the following possible cycle: hsp 70ADP, either free or associated with a protein substrate, binds RF-hsp 70, is converted to hsp 70-ATP, then dissociates from RF-hsp 70, and finally undergoes ATP hydrolysis with dissociation from a protein substrate and formation of free hsp 70-ADP. Although RF-hsp 70 may not be absolutely required for hsp 70 recycling, our results strongly suggest that efficient recycling of hsp 70 is dependent upon RF-hsp 70 action. The characteristics of RF-hsp 70 suggest that its function may be analogous to activities termed DnaJ and GrpE in bacteria (15 and YD) 1 in yeast (16), which stimulate the ATPase activity of the corresponding hsp 70, increase the rate of release of bound ATP or ADP from hsp 70 (GrpE), and enhance dissociation of hsp 70 from unfolded protein in the presence of ATP (YD1). As
we will show in the following article, however, the primary structure of RF-hsp 70 is not similar to that of these proteins. Instead, it shows appreciable sequence similarity to the yeast protein STI1 (31, 32), a protein that is increased with heat stress but whose function is currently unclear (31).

We wish to stress that, although we believe RF-hsp 70 has an important role in promoting the folding or chaperonin activity of hsp 70, we do not believe it is the only activity required for optimal hsp 70 function. For example, although RF-hsp 70 increases the rate of renaturation of heat-denatured luciferase by hsp 70 up to 10-fold in an isolated reaction in the absence of other protein factors, it does not fully reconstitute the activity of the intact reticulocyte lysate (Table I and Refs. 18 and 27). One additional participant in the folding reaction mediated by hsp 70 is hsp 90, which enhances the renaturation of luciferase in the presence of hsp 70 (18) but is not as effective as RF-hsp 70 (Fig. 8). Two other possible candidates are hsp 40, a mammalian homolog to bacterial DnaJ (33, 34), and the TCP-1 ring complex that is analogous to bacterial GroEL or hsp 60 (35). Both of these activities have been shown recently to participate with hsp 70 in the folding of newly synthesized luciferase in rabbit reticulocyte lysate (36). In addition, the renaturation of denatured luciferase using fractions from rabbit reticulocyte lysate separated by size appeared to be associated with TCP-1 in a study by Nimmesgern and Hartl (27) but not in the study by Schumacher et al. (18). One possible basis for this difference is that in the former study (27), luciferase was denatured completely with 6 M guanidinium chloride; whereas in the latter (18) and in our experiments above, luciferase was inactivated with mild heat and probably was not denatured completely. It is then possible that folding and activation of fully denatured (27) or newly synthesized (36) luciferase may be dependent upon TCP-1, whereas the reactivation of heat-denatured luciferase (18) may not. Clearly, further investigation will be required to resolve this point as well as to determine more precisely what activities in addition to RF-hsp 70 are required for optimal chaperonin function by hsp 70.

We have also observed partial specificity in the ability of RF-hsp 70 to stimulate the ATPase and chaperonin activities of different hsp 70s. RF-hsp 70 is most effective with its own hsp 70, hsp 70(R). RF-hsp 70 is less effective with hsp 70(B), even less effective with hsp 70(H), and ineffective with hsp 70(bn). These differences presumably reflect differences in the primary structure of the various hsp 70s. Although the complete primary structure of hsp 70(R) is not currently known, we have determined the sequence of its carboxyl-terminal 35 residues2 and found this to be identical to that of the constitutively expressed rat hsp 70 (19, 20) and to differ from that of the constitutively expressed hsp 70(B) (37) at only one site. This has verified that hsp 70(R) is the constitutively expressed form of hsp 70, and it suggests that the partial specificity observed could correlate with how close the primary structure of each hsp 70 is to hsp 70(R). The hsp 70(B) may be most similar, hsp 70(H) is probably more divergent since it is the inducible form of hsp 70 with a very different carboxyl-terminal sequence (38), and hsp 70(bn) is probably the most divergent.

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