We reported recently that a rabbit reticulocyte 66-kDa protein (termed RF-hsp 70 by our laboratory and p60 and hop by others) functions as a hsp 70 recycling protein and markedly enhances the renaturation of luciferase by hsp 70 (Gross, M., and Hessefort, S. (1996) J. Biol. Chem. 271, 16833-16841). In this report, we confirm that the ability of RF-hsp 70 to promote the conversion of hsp 70-ADP to hsp 70-ATP, thus enhancing the protein folding activity of hsp 70, is caused by the purified 66-kDa protein and not by a trace DnaJ/hsp 40 protein contaminant. To determine the relationship between RF-hsp 70 and the DnaJ/hsp 40 heat shock protein family, which also enhances protein renaturation by hsp 70, we purified a 38-kDa protein from rabbit reticulocyte lysate based upon its ability to stimulate late renaturation of luciferase by hsp 70. Partial amino acid sequencing of this 38-kDa protein has indicated, unexpectedly, that it is the enzyme δ-aminolevulinic acid dehydratase (ALA-D) and that it does not contain detectable sequences corresponding to the DnaJ/hsp 40 protein family. In addition, immunoblot analysis with a polyclonal antibody made to HeLa cell hsp 40 (from StressGen) confirms that our purified ALA-D contains no hsp 40, although hsp 40 is present in relatively crude rabbit reticulocyte protein fractions. Rabbit reticulocyte ALA-D is active in converting δ-aminolevulinic acid to porphobilinogen and as Zn²⁺-dependent as ALA-D purified from other sources. Rabbit reticulocyte ALA-D stimulates the renaturation of luciferase by hsp 70 up to 10-fold at concentrations that are the same as or less than that of hsp 70, and it has no renaturation activity in the absence of hsp 70. The renaturation effect of ALA-D is additive with that of RF-hsp 70 at limiting or saturating concentrations of each, and, unlike RF-hsp 70, ALA-D does not promote the dissociation of hsp 70-ADP in the presence of ATP. The renaturation-enhancing effect of ALA-D may be caused by a region near its carboxyl terminus which has sequence homology to the highly conserved domain of the DnaJ protein family, which is similar to the sequence homology between this domain and a carboxyl-terminal region in auxilin, a DnaJ-like protein that requires this region for its hsp 70-dependent function (Ungewickell, E., Ungewickell, H., Holstein, S. E. H., Lindner, R., Prasad, K., Barouch, W., Martin, B., Greene, L. E., and Eisenberg, E. (1995) Nature 378, 632-635).

We reported recently the purification of a 66-kDa protein from rabbit reticulocyte lysate which is found associated with hsp 70; we termed it RF-hsp 70 because it promotes the recycling of hsp 70 by stimulating the dissociation of ADP from, and the binding of ATP to, hsp 70 (1). Consistent with a recycling function, RF-hsp 70 increases by 10-fold the rate of renaturation of heat-denatured luciferase by hsp 70 (1). Extensive amino acid sequence analysis of RF-hsp 70 demonstrated (2) that it is structurally homologous to human IEF SSP 3521 (3), mouse extendin (4), and chicken p60 (5), suggesting that these proteins may also function to recycle hsp 70. However, when recombinantly expressed (in bacteria) IEF SSP 3521 was tested, it failed to enhance the renaturation of luciferase (6) or β-galactosidase (7) by hsp 70, in contrast to the activity of RF-hsp 70. Further testing of the recombinant human homolog of RF-hsp 70 (termed hop) by Johnson et al. (8) showed that although it does bind to hsp 70 and to hsp 90 and does stimulate luciferase renaturation in the presence of hsp 70 and YDJ-1 (a yeast DnaJ/hsp 40 homolog) or hsp 70, YDJ-1, and hsp 90, it is inactive with hsp 70 alone, in contrast to our previous findings (1). Furthermore, the recombinant hop failed to stimulate the ATPase activity of hsp 70, the dissociation of hsp 70-ADP, and the binding of ATP to hsp 70 (8), all in contrast to our report (1). In addition, several recent reports have demonstrated that members of the DnaJ/hsp 40 protein family, YDJ-1 and Hdj-1, do promote, respectively, the renaturation of luciferase (9) and β-galactosidase (7, 10) by hsp 70, similar to the effect of RF-hsp 70. Because of these contrasting findings and the finding that trace amounts of DnaJ/hsp 40 protein can contaminate other proteins involved in the renaturation process and influence their activity (9, 11), we tested and will show that there is no detectable, silver-stained protein migrating further than the 66-kDa protein on SDS-polyacrylamide gel electrophoresis, even when a 10-fold excess of purified RF-hsp 70 is analyzed, and that RF-hsp 70 contains no hsp 40 as determined by immunoblot analysis with antibody to hsp 40.

To determine the relationship between the effects of RF-hsp 70 and the DnaJ protein family, we purified a protein from rabbit reticulocyte lysate which stimulates the hsp 70-mediated renaturation of denatured luciferase by a mechanism that is distinct from that of RF-hsp 70. This protein migrates as a
38-kDa band on SDS-polyacrylamide gel electrophoresis, the size characteristic of the DnaJ protein family. Partial amino acid sequence analysis of this protein has demonstrated, however, that it is the enzyme \( \delta \)-aminolevulinic acid dehydratase (ALA-D), and immunoblot analysis has confirmed that it is free of any hsp 40. In addition, enzymatic assay has demonstrated that the purified 38-kDa protein converts \( \delta \)-aminolevulinic acid (ALA) to porphobilinogen (PBG). We will show that the ability of this protein to enhance renaturation of luciferase by hsp 70 may be caused by a region near its carboxyl terminus which has considerable sequence homology to the highly conserved J domain characteristic of the DnaJ protein family. In this regard, the protein folding enhancement we have observed with ALA-D may be similar to the role that auxilin is believed to play in the hsp 70-mediated uncoating of reconstituted clathrin baskets (12, 13). Auxilin action is believed to be mediated by a region near its carboxyl terminus which shows the same degree of homology to the DnaJ consensus sequence (12, 13) which we have found in ALA-D.

**EXPERIMENTAL PROCEDURES**

**Purification of Protein Components**—The purification of hsp 70 from rabbit reticulocyte lysate was as described previously (14), and its purity is determined in Fig. 1B (lane 9). Its migration upon SDS-polyacrylamide gel electrophoresis and sequencing of a 35-amino acid polypeptide containing its carboxyl terminus, obtained by limited chymotrypsin digestion, indicate that this hsp 70 is the constitutively expressed form or hsc 73 (1). Unless otherwise indicated, this was the hsp 70 preparation used in all experiments. The purification of hsp 90 and RF-hsp 70 from rabbit reticulocyte lysate was as described (1), except that the final purification step (step 6) for RF-hsp 70 employed chromatography on a Superdex 200 HR 10/30 column (1.0 × 30 cm; Amersham Pharmacia Biotech). Step 5 RF-hsp 70 (0.50 ml and up to 4 mg/ml protein) was applied, and the column was equilibrated and eluted with 20 mM Tris–HCl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, and 0.1 mM EDTA at a flow rate of 0.50 ml/min using the Amersham Pharmacia Biotech fast protein liquid chromatography system. The absorbance at 280 nm was monitored, and 0.50-ml fractions were collected. This step 6 RF-hsp 70 appears homogeneous even when a large absorbance at 280 nm was monitored, and 0.50-ml fractions were collected.

To verify that the activity of RF-hsp 70 is attributable to its 66-kDa component, which is homologous to human IEF SSP 3521 (3), mouse extendin (4), and chicken p60 (5), and not to a possible DnaJ protein contaminant, we subjected a large amount of the 38-kDa protein to SDS-polyacrylamide-SLS slab gels (17) followed by silver staining (18) has been described previously.

**Materials**—Reagents for the renaturation and assay of firefly luciferase were all from Sigma, except that dithiothreitol was obtained from Calbiochem. All protein standards and ALA were purchased from Sigma, and dithiothreitol was obtained from Calbiochem. All protein standards and ALA were purchased from Sigma, except that dithiothreitol was obtained from Calbiochem. All protein standards and ALA were purchased from Sigma, except that dithiothreitol was obtained from Calbiochem. All protein standards and ALA were purchased from Sigma, except that dithiothreitol was obtained from Calbiochem.

**RESULTS**

**Purification of a Rabbit Reticulocyte Protein, Distinct from RF-hsp 70, Which Stimulates the Renaturation of Luciferase by Hsp 70**—To verify that the activity of RF-hsp 70 is attributable to its 66-kDa component, which is homologous to human IEF SSP 3521 (3), mouse extendin (4), and chicken p60 (5), and not to a possible DnaJ protein contaminant, we subjected a large excess of several step 6 RF-hsp 70 samples to SDS-polyacrylamide gel electrophoresis and silver staining. The results (Fig. 1A) demonstrate that these preparations contain no protein migrating faster than the 66-kDa band, indicating that they are devoid of a DnaJ protein member, which would migrate at about 40 kDa. In addition, immunoblot analysis of an excess of steps 5 and 6 RF-hsp 70, using a polyclonal antibody made against HeLa cell hsp 40 (see Fig. 3, lanes 10 and 8, respectively), confirms that these preparations are devoid of any hsp 40.

To investigate the relationship between the action of RF-hsp 70 and the DnaJ protein family, we attempted to purify the corresponding DnaJ protein from rabbit reticulocyte lysate. Using the stimulation of the renaturation of heat-denatured luciferase by hsp 70 as our assay, we purified a relatively abundant protein that migrates as a 38-kDa band on a denaturing gel, a size that is consistent with a DnaJ protein family member. The purification of this protein, based upon its ability to enhance hsp 70-mediated renaturation of luciferase, is
shown in Table I, under “Luciferase renaturation,” and gel analysis of the protein at different steps in the purification is shown in Fig. 1B. The results indicate that this 38-kDa protein is purified to near homogeneity at step 5 (Mono Q) and to apparent homogeneity at step 6 (Superdex 200), as seen in Fig. 1B, lanes 6 and 7, respectively. For comparison, purified RF-hsp 70 and hsp 70 have been run in lanes 8 and 9, respectively (Fig. 1B). We chose to purify the 38-kDa protein from the pH 5.2 precipitate and 0–40% ammonium sulfate fractions (Fig. 1B, lanes 2 and 3, respectively) because the pH 5.2 soluble fraction (not shown) and the 40–80% ammonium sulfate fraction (Fig. 1B, lane 4) have virtually no 38-kDa protein. In contrast, RF-hsp 70 is found almost entirely in the 40–80% ammonium sulfate fraction, which was used for its purification (1).

The results in Table I, “Luciferase renaturation,” indicate that the 38-kDa protein, based upon its ability to enhance the renaturation of luciferase by hsp 70, has been purified 29-fold from the ammonium sulfate fraction (step 3) with a yield of 12%. We are not able to determine the activity of the 38-kDa protein in steps 1 and 2 because they also contain RF-hsp 70.

The 38-kDa Protein Is ALA-D—We had presumed that the 38-kDa protein may be a rabbit reticulocyte lysate DnaJ protein (hsp 40) member because of its size, relative abundance (an estimated concentration of 0.1 mg/ml as indicated below), and ability to enhance luciferase renaturation by hsp 70. However, when we subjected the purified 38-kDa protein to NH₂-terminal sequence analysis, we obtained a single amino acid sequence (20 residues) that corresponds to the protein ALA-D (see Fig. 2), the enzyme (EC 4.2.1.24) that catalyzes the synthesis of PBG from two molecules of ALA. The sequence of the NH₂-terminal 20 amino acid residues of the rabbit reticulocyte 38-kDa protein is identical to the translated cDNA sequence of human ALA-D at all but the 18th residue, where serine replaces alanine, as is the case with mouse ALA-D. Because this finding was unexpected, we subjected the purified 38-kDa protein to trypsin digestion, peptide separation (by reverse phase chromatography), and additional amino acid sequence analysis to determine whether the 38-kDa preparation might also contain a known DnaJ protein, whose size is identical to that of ALA-D but whose amino terminus is blocked. The sequences of three of these tryptic peptides corresponded to residues 18–39, 61–74, and 91–118 in ALA-D (Fig. 2), confirming that the purified 38-kDa component is ALA-D and, apparently, only ALA-D. Sequencing of 81 of a total of 330 residues demonstrates that the rabbit reticulocyte protein has 88% identity to human and mouse ALA-D (Fig. 2).

Careful examination of the sequencing analyses of the 38-kDa protein revealed no detectable polypeptides corresponding to the DnaJ/hsp 40 protein family, although we did detect small amounts of two polypeptides derived from the added trypsin. Nevertheless, other laboratories have found that small amounts of the DnaJ/hsp 40 protein family may contaminate preparations of other proteins that promote protein folding and renaturation and contribute to their effect (9, 11). Therefore, we used polyclonal antibody, raised to HeLa cell hsp 40 (19) and obtained from StressGen, and immunoblot analysis (20) to try to verify that the renaturation effect of the purified 38-kDa protein (Table I “Luciferase renaturation”) is caused by ALA-D and not contaminating hsp 40. This analysis (Fig. 3) demonstrated that relatively crude fractions (steps 1–3, 38 kDa) do contain hsp 40. However, this hsp 40 is almost completely removed in the preparation of step 4, 38 kDa, and it is totally absent in steps 5 and 6, 38 kDa, demonstrating that the renaturation effect of the purified 38-kDa protein is not caused by contaminating hsp 40 and confirming that it is the result of ALA-D itself. Comparison of the immunoblot (Fig. 3) with a silver-stained gel run in parallel showed that ALA-D migrates slightly further than hsp 40, consistent with the fact that ALA-D is 330 amino acid residues in length, whereas hsp 40 is 340 (21).

Although we have shown that it is rabbit reticulocyte ALA-D that promotes the renaturation of luciferase by hsp 70 (Table I), an activity that is characterized in greater detail below, it was important to determine whether the same protein is also enzymatically active. The results in Table I indicate that the same fractions that promote renaturation of luciferase by hsp 70 also}

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**Protein Renaturation and δ-Aminolevulinic Acid Dehydratase**

**FIG. 1.** Gel electrophoretic analysis of an excess of purified RF-hsp 70 and the purification steps of a rabbit reticulocyte 38-kDa protein. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis and then silver staining as described under “Experimental Procedures.” Panel A: lanes 1–3, 2, 1.5, and 2 μg of three different preparations of purified (step 6) RF-hsp 70. Panel B: lane 1, 200 μg of step 1, 38-kDa protein (post-ribosomal supernatant); lane 2, 30 μg of step 2, 38-kDa protein; lane 3, 28 μg of step 3, 38-kDa protein (0–40% ammonium sulfate fraction from step 2); lane 4, 19 μg of 40–80% ammonium sulfate fraction from step 2, 38-kDa protein; lane 5, 3.2 μg of step 4, 38-kDa protein; lane 6, 0.3 μg of step 5, 38-kDa protein; lane 7, 0.25 μg of step 6, 38-kDa protein; lane 8, 0.2 μg of purified (step 6) RF-hsp 70; lane 9, 0.2 μg of purified, rabbit reticulocyte hsp 70. The arrows on the left indicate the migration positions of hsp 70 (73 kDa), RF-hsp 70 (66 kDa), and the 38-kDa protein; the arrows on the right mark the migration positions of protein standards that were run in a parallel lane. Electrophoresis was at 44 V (3 V/cm) for 15 h in panel A and 20 h in panel B.
TABLE I
Purification of a rabbit reticulocyte 38-kDa protein (ALA-D)

| Step | Volume | Concentration | Luciferase renaturation | ALA conversion to PBG |
|------|--------|---------------|-------------------------|-----------------------|
|      | ml     | mg/ml         | -fold | % | Purification | Yield | Units/ml | Units/mg | -fold | % | Purification | Yield |
| 1. Supernatant | 1,150 | 135 | 43 | 1.0 | 100 | 0.79 | 0.0058 | 1.0 | 100 |
| 2. pH 5.2 precipitate | 383 | 30 | 43 | 1.0 | 100 | 3.0 | 0.10 | 17 | 126 |
| 3. Ammonium sulfate | 61 | 78 | 0.10 | 1.0 | 13 | 18.3 | 0.23 | 0.2 | 123 |
| 4. DEAE-cellulose | 48 | 13 | 2.00 | 153 | 36 | 47 | 10.6 | 0.82 | 141 | (3.5) | 56 |
| 5. Mono Q | 39 | 1.0 | 100 | 1,100 | 26 | 21 | 8.0 | 8.0 | 1,380 (35) | 34 |
| 6. Superdex 200 | 13.5 | 1.5 | 1,860 | 1,240 | 29 | 12 | 17.2 | 11.5 | 1,960 (50) | 25 |

FIG. 2. Partial amino acid sequences of the 38-kDa protein are virtually identical to the translated sequences of the cDNA of human, rat, and mouse ALA-D. The amino acid sequence of the first 150 residues of human ALA-D, determined from the cDNA as reported by Ishida et al. (46), is shown on each top line. Under this are aligned the corresponding sequences of rat (47) and mouse (48) ALA-D, as determined from the respective cDNA, and then partial sequences derived from NH$_2$-terminal sequencing of the intact, purified 38-kDa protein and three major polypeptides isolated from this rabbit reticulocyte protein after trypsinization and reverse phase chromatography as described under "Experimental Procedures." The single-letter code for the amino acids has been used, and an asterisk denotes a residue that is identical to that of human ALA-D. Only the amino-terminal 45% of this 330-amino acid protein is shown because all rabbit 38-kDa protein sequences determined were virtually identical to the translated sequences of the cDNA of human, rat, and mouse ALA-D. The purification and yield of ALA-D are probably appreciable less than the 1980-fold and 25%, respectively, shown in Table I, "ALA conversion to PBG," as explained above. Taking this into account, we estimate that the concentration of ALA-D in rabbit reticulocyte lysate is on the order of 0.1 mg/ml, similar to our estimate of RF-hsp 70 in reticulocyte lysate (1).
ular weight, based upon density gradient sedimentation or gel filtration analysis, of 285 kDa (bovine liver) (26), 250 kDa (Rhodobacter spheroides) (27), or 252 kDa (human erythrocyte) (23). Chromatography of rabbit reticulocyte ALA-D on a calibrated Superdex 200 column (data not shown) indicates an apparent native molecular mass of about 275 kDa, very similar to that of the other reported ALA-D species.

Our initial assays of the enzymatic activity of ALA-D (Table 1) were performed with Tris-HCl buffer at pH 7.5 to match the pH and buffer used to measure the renaturation of luciferase by hsp 70 and its enhancement by ALA-D and RF-hsp 70 (examined in detail below). We also measured ALA-D enzymatic activity in sodium phosphate buffer at pH 6.5, the approximate pH optimum reported for crude rat liver ALA-D (16) or ALA-D purified from bovine liver (28) or human erythrocytes (23), to permit comparison of the specific activity of the purified rabbit reticulocyte enzyme with that of ALA-D from these other sources. Unexpectedly, we found that rabbit reticulocyte ALA-D has optimal enzyme activity at pH 7.5–8.5, whereas it has only about 40% and 10% of optimal activity at pH 6.5 and 6.0, respectively (Fig. 5). When the pH dependence of enzyme activity was tested with Tris-HCl in place of sodium phosphate, the pH optimum and level of enzyme activity were very similar (Fig. 5), indicating that our result is not peculiar to a specific buffer. The difference in pH optimum observed is more likely caused by a difference in species than in tissue of origin because the pH optima of human erythroid and rat and bovine liver enzymes are similar, whereas that of rabbit erythroid ALA-D differs. It is of interest that the pH optimum for rabbit reticulocyte ALA-D is rather similar to that of ALA-D isolated from R. spheroides (27). When comparison is made at the respective optimal pH, the specific activity of purified rabbit reticulocyte ALA-D of 11.5 units/mg compares fairly well with 18 units/mg for the purified human erythrocyte enzyme (23) and 23 units/mg for bovine liver ALA-D (24).

Characterization of the Renaturation Effect of Rabbit Reticulocyte ALA-D—The effects of limiting and saturating concentrations of purified rabbit reticulocyte ALA-D and RF-hsp 70, added separately and together, on the renaturation of heat-denatured luciferase by hsp 70 are shown in Fig. 6. In this and other such experiments, heat treatment reduced luciferase activity to about 6% of the initial activity, and reincubation of
luciferase by itself resulted in virtually no renaturation (Figs. 6 and 7). The concentration of added hsp 70 was 1.0 mM, which we (1) and Schumacher et al. (6) previously found was just saturating in this renaturation assay. Incubation was for 60 min, the time where renaturation approaches a maximum (see Fig. 7). The results (Fig. 6) show that little renaturation occurs with hsp 70 alone because luciferase activity increased from 8.5% to only 10.5% of its initial activity before heat denaturation. In contrast, ALA-D produced a dose-dependent increase in the renaturation of luciferase in the presence of hsp 70 (and absence of RF-hsp 70) which reached a maximum of just more than 30% of the initial luciferase activity. Although the maximal effect of ALA-D is much less than complete (100%) renaturation, it does represent a more than 10-fold increase in renaturation over that produced by incubation with hsp 70 alone. The renaturation effect of ALA-D is completely dependent upon the presence of hsp 70 because ALA-D has no effect when hsp 70 is not added (Fig. 6 and Table II). Thus, as is the case with RF-hsp 70 (1), ALA-D appears to promote protein renaturation by enhancing the folding or chaperonin activity of hsp 70. The action of ALA-D is not a nonspecific effect of adding more protein because the same amount of bovine serum albumin or glutathione S-transferase produces no stimulation of luciferase renaturation (Table II). The concentration of ALA-D which is just saturating, 60 μg/ml (Fig. 6), corresponds to 1.6 μM relative to the 38-kDa monomer or 0.2 μM for the octamer, indicating that at saturation, ALA-D enhances the renaturation activity of hsp 70 at a molar concentration that is about equal to or less than that of hsp 70.

For comparison, the effect of limiting and saturating amounts of RF-hsp 70 on the renaturation of luciferase in the presence of hsp 70 and absence of ALA-D is graphed on the ordinate of Fig. 6. RF-hsp 70 produced a just saturating stimulation of renaturation of about 60% of the initial luciferase activity at 1.0 μM, the same concentration as the added hsp 70, as we reported previously (1). When added at 0.25 μM, RF-hsp 70 promoted renaturation to just under 30% of the original luciferase activity, similar to the effect of saturating ALA-D. When ALA-D and RF-hsp 70 were added together to renaturation reactions, they produced exactly an additive effect on luciferase renaturation, whether each was added at a limiting or saturating concentration (Fig. 6). Thus, for example, just saturating concentrations of RF-hsp 70 and ALA-D promoted luciferase renaturation to 56 and 31% of its initial activity, respectively, when added separately, and they promoted renaturation to 84% of the original luciferase activity when added together. This indicates that the effect of each on luciferase renaturation by hsp 70 is independent of the other and is probably mediated by separate mechanisms.

We also examined the effect of just saturating levels of ALA-D and RF-hsp 70 on luciferase renaturation by hsp 70 as a function of time. The results (Fig. 7) indicate that renaturation of luciferase in the presence of ALA-D, RF-hsp 70, or both

![Fig. 6. Effect of ALA-D and RF-hsp 70 on the renaturation of heat-denatured luciferase by hsp 70. Renaturation reactions (25 μl), prepared and incubated as indicated under "Experimental Procedures," contained the indicated concentrations of purified ALA-D and 0.0 (×), 0.14 (●), 0.25 (○), 0.50 (◇), 1.0 (▲), or 1.5 (△) μM RF-hsp 70. All samples also contained 1.0 μM hsp 70 except those depicted with an ×, which received no added hsp 70. Luciferase activity was measured after a 60-min incubation as described under "Experimental Procedures" and is expressed as a percentage of the activity in luciferase samples that were not heat-denatured. Samples of heat-denatured luciferase that were not reincubated had 6.6% of the initial activity. The results are a composite of four separate experiments.](image1)

![Fig. 7. Effect of ALA-D, RF-hsp 70, or both on the rate of renaturation of heat-denatured luciferase by hsp 70. Renaturation reactions contained 60 μg/ml ALA-D (●, +), 1.0 μM RF-hsp 70 (○, +), or neither protein (○, ●). All samples but (○) also received 1.0 μM hsp 70. The final volume was 110 μl, and 25-μl aliquots were removed at the indicated times for the determination of luciferase activity. Heat-denatured luciferase that was not reincubated had 6.5% of the initial activity of luciferase samples that had not been heated. The results are at average of two separate experiments.](image2)
Duplicate samples of heat-denatured luciferase were incubated for 60 min under conditions of renaturation (final volume, 25 μl) and then assayed for luciferase activity as indicated under “Experimental Procedures.” Unless otherwise indicated, final concentrations were 1.0 μM hsp 70, 60 μg/ml ALA-D, 0.65 μM RF-hsp 70, 60 μg/ml bovine serum albumin, 60 μg/ml glutathione S-transferase, 1.0 μM hsp 90, and 3.0 μM ZnCl₂. Added hsp 70 was rabbit reticulocyte unless otherwise indicated. In the column labeled ADP, renaturation incubations contained 0.5 mM ADP in place of ATP and received no creatine phosphate. All values are an average of at least two experiments, and most are an average of three. ND = values that were not determined. Luciferase that was heated and not reincubated had an average of 6.2% of the activity before heating (range, 5.2–7.1%).

| Hsp 70 plus | Percent of initial luciferase activity | Added Hsp 70 | Hsp 90 | ZnCl₂ | ADP | −ALA-D | +ALA-D | Percent of initial luciferase activity |
|-------------|---------------------------------------|-------------|--------|-------|-----|-------|-------|-------------------------------------|
| No hsp 70 added | 9.0 | ND | ND | 8.0 | None | 8.6 | 8.4 | |
| ALA-D (30 μg/ml) | 11.7 | 14.4 | 13.3 | 13.3 | Rabbit reticulocyte | 10.7 | 23.8 | |
| ALA-D | 18.1 | 16.3 | ND | ND | Bovine brain | 10.2 | 17.2 | |
| RF-hsp 70 | 24.1 | 22.1 | 25.4 | 14.1 | Human (hsp 72) | 11.0 | 13.4 | |
| Bovine serum albumin | 37.4 | 34.5 | 39.7 | 12.9 | | | | |
| Glutathione S-transferase | 11.1 | ND | ND | ND | | | | |

is incubation time-dependent. Neither (nor both together) increased the activity of heat-denatured luciferase when added without incubation, indicating that their effect is at the level of protein renaturation or folding. The effects of ALA-D and RF-hsp 70 were about additive at every time point, producing renaturation of luciferase of 26% (ALA-D), 70% (RF-hsp 70), and 85% (both) of the initial activity by 110 min (Fig. 7). Whereas renaturation with ALA-D reached a maximum at 70 min, renaturation in samples with RF-hsp 70 was still increasing at 110 min. Results in Figs. 6 and 7 demonstrate that saturating ALA-D and RF-hsp 70 together can produce almost complete renaturation of luciferase by hsp 70. In contrast, incubation of heat-denatured luciferase by itself or with only hsp 70 added produces very little renaturation of activity.

We found previously that increasing the hsp 70 concentration above 1.0 μM produces little further increase in luciferase renaturation by saturating RF-hsp 70 but that below 1.0 μM, hsp 70 becomes inhibitory (1). Therefore, we compared the dependence upon hsp 70 concentration of luciferase renaturation in the presence of ALA-D with that in the presence of RF-hsp 70 (Fig. 8). Renaturation in the presence of limiting (0.3 μM) RF-hsp 70 requires at least 1.0 μM hsp 70 for a near maximal effect and is only 62 and 23% as great, respectively, with 0.50 and 0.25 μM hsp 70 (Fig. 8B). In contrast, renaturation in the presence of limiting ALA-D is almost as great with 0.25 μM hsp 70 as it is with higher hsp 70 concentrations, and even when ALA-D is saturating, renaturation is still 63% as great with 0.25 μM hsp 70 as it is with 1.0 μM hsp 70 (Fig. 8, A and B). Thus, limiting RF-hsp 70 (0.3 μM) is somewhat more effective at stimulating luciferase renaturation than saturating ALA-D at 1.0 μM hsp 70 or above, but it is only one-half as effective at 0.25 μM hsp 70 (Fig. 8B).

Additional characterization of the effect of ALA-D on the renaturation of luciferase by hsp 70 is shown in Table II. When we tested the action of ALA-D and RF-hsp 70 on renaturation with ADP added in place of ATP and an ATP-regenerating system, we found that neither produced any stimulatory effect, in contrast to their activity in the presence of ATP, confirming that the action of each requires ATP. We also tested and found that hsp 90, which has a small stimulatory effect on renaturation by hsp 70, does not enhance renaturation in the presence of limiting ALA-D or RF-hsp 70 or just saturating ALA-D (Table II). Other studies have shown that hsp 90 maintains denatured protein in a folding-competent state (7, 9, 10). In contrast to the dependence upon Zn²⁺ of ALA-D enzymatic activity (Fig. 4), the renaturation effect of just saturating ALA-D is not significantly enhanced by Zn²⁺ (Table II). This finding is consistent with the possibility that these two activities of ALA-D are mediated by different parts of the protein.

Finally, we tested and found that the degree of renaturation effect produced by ALA-D is dependent upon the specific hsp 70 added (Table II) because it is most effective with its own (rabbit reticulocyte) hsp 70 (hsc 73 or constitutive form) but only about one-half as effective with bovine brain hsp 70 (constitutive form) and less than one-fifth as effective with human hsp 70 (heat-inducible form). We reported previously a similar pattern of hsp 70 specificity with RF-hsp 70 (1).

To determine whether the mechanism by which ALA-D enhances renaturation by hsp 70 is different from that of RF-hsp 70, we tested the ability of each or both to promote the dissociation of hsp 70-ADP in the presence of ATP. We showed previously that RF-hsp 70 binds to hsp 70, lowers the K₆₅ of hsp 70 for ATP to a value that is close to its K₄₅ for ADP, and thus stimulates the conversion of hsp 70-ADP to hsp 70-ATP leading to enhanced hsp 70 recycling (1). The results in Fig. 9 demonstrate that the dissociation of hsp 70-[³²P]ADP in the presence of ATP is a relatively slow reaction, and it is not affected by the addition of limiting (30 μg/ml) or just saturating (60 μg/ml) concentrations of ALA-D. In contrast, this dissociation occurs about 4- and 5-fold faster in the presence of limiting (0.5 μM) and just saturating (1.0 μM) RF-hsp 70, respectively, and the stimulation by RF-hsp 70 is not affected by adding limiting or just saturating ALA-D (Fig. 9). We also tested and found that just saturating ALA-D has a relatively small effect on the ATPase activity of hsp 70 (it increases this activity 1.5-fold; data not shown) in contrast to the effect of just saturating RF-hsp 70, which stimulates the ATPase activity of hsp 70 up to 5-fold (1). These results confirm that RF-hsp 70 and ALA-D promote renaturation by hsp 70 by different mechanisms.

The Renaturation Stimulatory Activity of ALA-D May Be Caused by a Region Near Its Carboxyl Terminus Which Has Considerable Sequence Similarity to the Highly Conserved DnaJ Protein Family Domain—Although ALA-D is not a member of the DnaJ protein or hsp 40 family, we examined its primary structure to determine whether any part of it may show sequence homology to the DnaJ protein family. A precedent for this is the identification of the brain vesicle-associated protein auxinil as the 100-kDa cofactor that is required for hsp 70 (hsc 73)-dependent dissociation of clathrin baskets or clathrin release from coated vesicles (12, 13). The action of auxinil was found to be dependent upon a carboxyl-terminal domain of the protein (12) which contains considerable sequence similarity to the DnaJ domain, a highly conserved 70-amino acid region shared by all DnaJ-like proteins that otherwise show considerable sequence variation (12, 13). An examination of ALA-D demonstrates that it also contains a region near its carboxyl terminus which has considerable sequence similarity to the DnaJ protein family domain (Fig. 10). Two different
alignments (upper alone and lower plus right portion of upper) of human ALA-D (residues 259–296 and 232–296, respectively) with human Hdj-1 (residues 22–53 and 10–53, respectively) demonstrate 29% identity and 42% similarity between a region near the carboxyl terminus of ALA-D and the DnaJ domain of Hdj-1. These values are very close to the similarity between the carboxyl-terminal portion of auxilin and the DnaJ domain of HSJ1 (22% identity and 52% similarity) or a DnaJ consensus sequence (32% identity and 50% similarity) (13). They also are of the same magnitude as the similarity between bacterial DnaJ and the DnaJ consensus sequence (13). In addition, ALA-D contains the His-Pro-Asp sequence (at 268–270) that is present in all DnaJ proteins and has been found to be necessary for the function of DnaJ (29), yeast Sec63p (30), and yeast YDJ-1 (31). These findings suggest that the region near the carboxyl terminus of ALA-D may be a DnaJ domain and that the ability of ALA-D to promote the folding activity of hsp 70, as shown above, may be caused by an ability to function as a DnaJ protein.

**DISCUSSION**

This study was prompted in part by the difference between our characterization of RF-hsp 70, purified from rabbit reticulocyte lysate (1), and the characterization of the homologous human protein, IEF SSP 3521 (3), expressed as a recombinant protein in, and purified from, Escherichia coli and termed p60 or hop (6–8). We found that RF-hsp 70 stimulates the dissociation of ADP from, and the binding of ATP to, hsp 70, leading to a dramatic increase in the rate of renaturation of luciferase by hsp 70 (1 and this report). In contrast, Johnson et al. (8) found that recombinant hop lacks these very same activities, although it does bind to hsp 70 and hsp 90, and it does enhance renaturation of luciferase somewhat if YDJ-1 or YDJ-1 and hsp 90 are also added. One possible explanation for this difference, raised by Johnson et al. (8), is that our RF-hsp 70 may contain contaminants and that hop/RF-hsp 70 itself does not recycle hsp 70 by promoting adenine nucleotide exchange. In particular, hop 40 has been found to contaminate preparations of other proteins involved in protein renaturation/folding (9, 11), and members of the DnaJ/hsp 40 protein family do stimulate protein renaturation by hsp 70 (7, 9, 10). Therefore, we tested and found that our RF-hsp 70 is completely free of hsp 40 (by immunoblot analysis in Fig. 3) and of trace amounts of any protein migrating faster than the 66-kDa band of RF-hsp 70 (by loading excess protein on the gel and silver staining in Fig. 1A). These results demonstrate convincingly that RF-hsp 70 does
have the properties of a hsp 70-recycling protein, as we showed previously (1) and in this report. An alternative explanation, suggested by Johnson et al. (8) and by us (1), is that, in contrast to the native cellular protein, recombinant hop/RF-hsp 70 may be defective, perhaps because this protein, when synthesized in E. coli, is not folded properly and/or does not undergo proper disulfide bond formation that may be required for its function. We believe this alternative remains a distinct possibility. Johnson et al. (8) also commented that the fact that hop/RF-hsp 70 binds to hsp 70-ADP and not to hsp 70-ATP (1, 8) is inconsistent with this protein acting as a nucleotide exchange factor, as we have proposed (1 and this article). We believe, however, that hop/RF-hsp 70 binding to hsp 70-ADP is precisely what would be expected for a recycling factor that acts by promoting adenine nucleotide exchange. As discussed previously (1), RF-hsp 70 binding to hsp 70-ADP, either free or associated with a protein substrate, increases the affinity (lowers the $K_d$) of hsp 70 for ATP, leading to a more rapid rate of dissociation of ADP from, and binding of ATP to, hsp 70 (1). This would increase the rate of recycling of hsp 70, leading to an increased rate of protein (luciferase) renaturation as observed (1 and this report) and result, secondarily, in an increased rate of conversion of ATP to ADP (ATPase activity).

We have also found that rabbit reticulocyte ALA-D, which is about as active in converting ALA to PBG as is ALA-D from other sources, has the additional ability to promote protein renaturation by hsp 70. We have proposed that this additional function may be the result of a region near the carboxyl terminus of ALA-D which has sequence homology to the conserved domain of the DnaJ/hsp 40 protein family, suggesting that ALA-D may act as a DnaJ-like protein, similar to what has been proposed for the brain protein auxilin (12, 13). Our characterization of the renaturation effect of ALA-D is most consistent with this hypothesis, although an alternative explanation for this unexpected action of ALA-D is certainly possible. Like the human DnaJ homolog Hdj-1, which enhances protein renaturation in the presence of hsp 70 but has no renaturing effect by itself (7, 10), ALA-D stimulates protein renaturation by hsp 70 and is ineffective by itself. Other DnaJ homologs, such as YDJ-1 (32) and E. coli DnaJ (for review, see Ref. 33), may have the additional ability to function as molecular chaperones themselves in the absence of the corresponding hsp 70. It is thought that this additional function requires the glycine/phenylalanine-rich and the cysteine-rich regions that are characteristic of only some DnaJ homologs (34). Unlike YDJ-1 and DnaJ, Hdj-1 and ALA-D lack these regions and possess only the conserved J domain. We have also found that the degree of renaturation produced by ALA-D is dependent upon the specific hsp 70 added (Table II). Previous studies with yeast proteins have also shown specificity in the cooperative action of individual hsp 70s and DnaJ homologs (32, 35, 36) and that this is determined by the structure of the individual J domain (36).

The folding or chaperone function of hsp 70 is regulated by specific DnaJ homologs, and this may be mediated by the direct interaction of the J domain of the DnaJ protein with a carboxyl-terminal portion of hsp 70 (34) which is different from the polypeptide binding domain of hsp 70 (33). Although the mechanism of how hsp 70 and DnaJ proteins function together to promote protein folding is not completely clear, studies using chaperone proteins from E. coli to renature thermally denatured luciferase in vitro by Schröder et al. (37) demonstrated that DnaK (hsp 70), DnaJ, and GrpE were sufficient to produce almost complete renaturation. They found that DnaJ associated with denatured luciferase and, together with DnaK, prevented luciferase aggregation, thus permitting subsequent reactivation. They also demonstrated that the same three proteins were necessary for renaturation in vivo. Ziemienowicz et al. (38) demonstrated that these same three proteins can reactivate heat-denatured RNA polymerase, that DnaJ reduced the level of DnaK required, and that both DnaJ and GrpE are involved in the formation and the dissociation of a substrate-chaperone complex.

We find it significant that these three E. coli proteins produce near complete renaturation in vitro, and we obtain almost complete renaturation with three rabbit reticulocyte proteins, one of which (hsp 70) is homologous to E. coli DnaK and another of which (ALA-D) may be homologous to E. coli DnaJ. The third, RF-hsp 70, although structurally related to yeast STI1 (2, 39), has appreciable functional similarity to E. coli GrpE. RF-hsp 70 promotes the release of ADP from hsp 70 in the presence of ATP (1, Fig. 9), and GrpE stimulates the release of ADP and ATP from DnaK (40). Although the combination of RF-hsp 70 and ALA-D promotes almost complete renaturation of heat-inactivated luciferase in the presence of hsp 70, the action of each with hsp 70 is not dependent upon the other (Figs. 6 and 7), and each functions by a different mechanism (Fig. 9). RF-hsp 70 promotes the recycling of hsp 70 by promoting adenine nucleotide exchange (1), whereas ALA-D, which may act as a DnaJ homolog, may promote the association as well as the dissociation of a hsp 70-substrate complex, as envisioned for other DnaJ proteins. One should also note that rabbit reticulocyte lysate may contain additional proteins that promote protein folding and renaturation, such as the large, hetero-oligomeric ring complex TCP-1 (hsp 60 homolog) (41). Similarly, E. coli proteins GroEL (hsp 60 equivalent) and GroES can reaggregate heat-denatured RNA polymerase by a mechanism that is separate from that of DnaK, DnaJ, and GrpE (38).

Schumacher et al. (9) found that 0.04—0.10 $\mu$M YDJ-1 produces a maximal stimulatory effect on luciferase renaturation in the presence of hsp 70, which is much lower than the concentration of ALA-D which is required for maximal effect (60 $\mu$g/ml or 1.6 $\mu$M relative to the 38-kDa monomer). Schumacher et al. (9) also reported that Hdj-1 was only 30% as effective as YDJ-1 in promoting renaturation. One explanation for these differences, as noted above and in Ref. 9, is that ALA-D and Hdj-1 lack the glycine- and phenylalanine-rich and cysteinerich domains that are present within YDJ-1 and other but not $\ldots$
all DnaJ proteins (34). Thus, Freeman and Morimoto (10) used 1.6 μM hsp 70 and 3.2 μM Hdj-1 for their renaturation reactions, concentrations that are similar to the concentrations (1.0 μM hsp 70 and 1.6 μM ALA-D) we have found to be optimal. One additional consideration is that, unlike other DnaJ proteins, such as E. coli DnaJ, which are thought to function as dimers (33), ALA-D is an octamer (23, 26, 27), which may require that it be added at a 4-fold greater molar concentration of the monomer to produce the same effect of the multimer.

Studies of the enzymes involved in heme biosynthesis have indicated that in most mammalian tissues, ALA-D is the enzyme whose concentration is in the greatest excess relative to the others in this pathway (42). Despite this, the level of ALA-D has been found to increase during erythroid cell differentiation. This suggests that ALA-D may be the major functional DnaJ protein in rabbit reticulocyte lysate, which is close to the concentration of hsp 70. We estimate that the concentration of ALA-D in rabbit reticulocyte lysate is approximately 0.1 mg/ml, which is close to the concentration of ALA-D (0.06 mg/ml) we have found to be just saturating in promoting the renaturation of luciferase by hsp 70 in an isolated reaction. Although rabbit reticulocytes do contain DnaJ-hsp 40 40 protein (9, Fig. 3), our findings suggest that this protein is much less abundant than ALA-D (data not shown). This suggests that ALA-D may be the major functional DnaJ protein in rabbit reticulocytes and that this may be produced in such great excess during erythroid cell differentiation.

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