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ATP-dependent protein refolding activity in reticulocyte lysate

Evidence for the participation of different chaperone components

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The protein folding capacity of rabbit reticulocyte cytosol was analyzed using the renaturation of firefly luciferase as a sensitive assay. In the absence of ATP, the aggregation of denatured luciferase diluted into reticulocyte lysate was prevented. Chaperone-stabilized luciferase was detected in high molecular weight complexes overlapping the distributions of Hsc70, Hsp90 and the chaperonin TRiC on gel filtration columns. The readdition of unfractionated cytosol and Mg-ATP was required for the efficient folding of these forms of luciferase to the active enzyme. We conclude that protein folding in the eukaryotic cytosol depends on the functional cooperation of different chaperone activities and cofactors in a complex, ATP-dependent process.

Protein folding; Molecular chaperone; Firefly luciferase

1. INTRODUCTION

The folding and oligomeric assembly of many if not most newly-synthesized proteins appears to be mediated by so-called molecular chaperone proteins [1,2]. These components function in preventing the incorrect inter- and intramolecular association of unfolded polypeptides that leads to their aggregation. Molecular chaperones of different classes can cooperate in a sequential protein folding pathway. For example, in the case of proteins which are imported from the cytosol into mitochondria as unfolded chains [3], folding requires an ordered pathway of interactions with the constitutively expressed heat-shock proteins (Hsp) 70 and 60 in the mitochondrial matrix, and the hydrolysis of ATP [4]. Similar reactions in the evolutionarily related cytosol of bacteria may involve the Hsp70 homolog DnaK, together with its chaperone partners DnaJ and GrpE, and the Hsp60 homolog GroEL [5,6]. By contrast, relatively little is known about the mechanisms and the components involved in protein folding in the eukaryotic cytosol. Ribosome-bound polypeptide chains have been shown to be associated with the cytosolic Hsp70 cognate (Hsc70) and this interaction is thought to be required for productive folding [7,8]. The eukaryotic cytosol also contains high concentrations of other molecular chaperones, such as Hsp90 [9], and the recently discovered TCP-1 ring-complex (TRiC). TRiC has been proposed to be the functional equivalent of Hsp60/GroEL in the cytosol [10–13].

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In an effort to identify the cytosolic components required for a complete protein folding reaction, we have developed an assay for the chaperone-dependent renaturation of unfolded firefly luciferase. This protein, although naturally located in specialized peroxisomes of the firefly [14], folds normally upon expression in the bacterial cytosol [15] and also in the cytosol of eukaryotic cells when peroxisomes are absent (J. Höhfeld and F.U.H., unpublished). Using this assay, we found that reticulocyte lysate is highly active in mediating ATP-dependent protein refolding. Our analysis revealed that protein folding in the eukaryotic cytosol is a complex reaction in which different chaperone activities participate.

2. MATERIALS AND METHODS

2.1. *Denaturation of luciferase*

Firefly luciferase (Sigma) was denatured at 10 μM for 1 h at 25°C in buffer A (6 M guanidinium-Cl (99.5% pure), 25 mM HEPES/KOH pH 7.5, 50 mM KOAc, 5 mM $Mg(OAc)_2$, 5 mM DTT). In some experiments, the protein was further diluted to 3.2 μM with buffer A before dilution into the refolding assay.

2.2. *Refolding Assay*

Denatured luciferase was diluted 100-fold into a 50 μl refolding assay containing buffer B (25 mM HEPES/KOH pH 7.5, 50 mM KOAc, 5 mM DTT) and other components where indicated in the figure legends. During incubation at 30°C, 1 μl aliquots were withdrawn at the indicated time points and added to 40 μl 5 × diluted luciferase assay mix (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP; Promega) using 25 mM Tris-SO_4, pH 7.8, 2 mM *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 1 mg/ml BSA as dilution buffer. After 10

s the samples were analyzed in a luminometer (Wallac) for 10 s. To obtain the native enzyme control, native luciferase was diluted into buffer C (buffer B containing 5 mM $Mg(OAc)_2$) with 1 mM ATP and the activity determined.

2.3 *Desalting of reticulocyte lysate*

Reticulocyte lysate (untreated lysate from Promega) was desalted on Nap5 columns (Pharmacia) equilibrated in buffer D (50 mM HEPES/KOH, pH 7.5, 100 mM KOAc, 5 mM DTT, 10% glycerol) or in buffer D containing 5 mM $Mg(OAc)_2$ where indicated. The protein-containing eluate was concentrated to the starting volume by Centricon 10 (Amicon) filtration

2.4. *Fractionation by gel-filtration*

2 μl denatured luciferase (10 μM) was diluted into 198 μl desalted lysate containing $Mg(OAc)_2$. After 15 min incubation at 25°C, the reaction was centrifuged for 10 min at 16,000 × *g*, 4°C. 10 μl of the supernatant were used for a refolding assay (addition of ATP to 5 mM) and 190 μl were loaded onto a Superose 6 column incubated in buffer D with 5 mM $Mg(OAc)_2$. After elution of the void volume (8.5 ml), 25 fractions of 0.5 ml were collected.

2.5. *Miscellaneous*

The following procedures were performed according to published methods: SDS-PAGE [16], electrotransfer of proteins onto nitrocellulose [17] and immunodetection using rabbit polyclonal antisera against luciferase and Hsc70 or monoclonal antibodies against TCP-1 (antibody 91A [18]) and Hsp90 (Affinity Bioreagents), and the ECL-system (Amersham) [19].

3. RESULTS

3.1. *ATP-dependent refolding of luciferase in reticulocyte lysate*

The 65 kDa protein firefly luciferase [20] was completely denatured in 6 M guanidinium-Cl (GdmCl), as judged by circular dichroism measurements (not shown), and then diluted 100-fold into buffer solution containing ATP. This resulted in a regain of only 6% of the enzyme activity after incubation for 40 min at 30°C (Fig. 1a). In contrast, when the denatured protein was diluted into buffer containing reticulocyte lysate and ATP, the enzyme efficiently refolded. The extent of reactivation was dependent on the final concentration of reticulocyte lysate in the reaction and approached an optimum at a lysate content of 40%, corresponding to a total protein concentration of ~70 mg/ml. The recovery of enzyme activity was then about 80%. These data suggested that the reticulocyte lysate either contains a molecular chaperone activity that supports the correct refolding of luciferase, or the high protein concentration in the refolding reaction is responsible for these effects. We found, however, that the control proteins bovine serum albumin (BSA) and haemoglobin were unable to support the efficient folding of luciferase. While at BSA and haemoglobin concentrations of 20–150 μg/ml the yield of active luciferase increased, at concentrations equivalent to those of total protein in the reticulocyte lysate, luciferase did not refold (Fig. 1b).

To investigate whether the observed refolding activity was indeed ATP-dependent, we removed nucleotides and other low molecular weight components from the

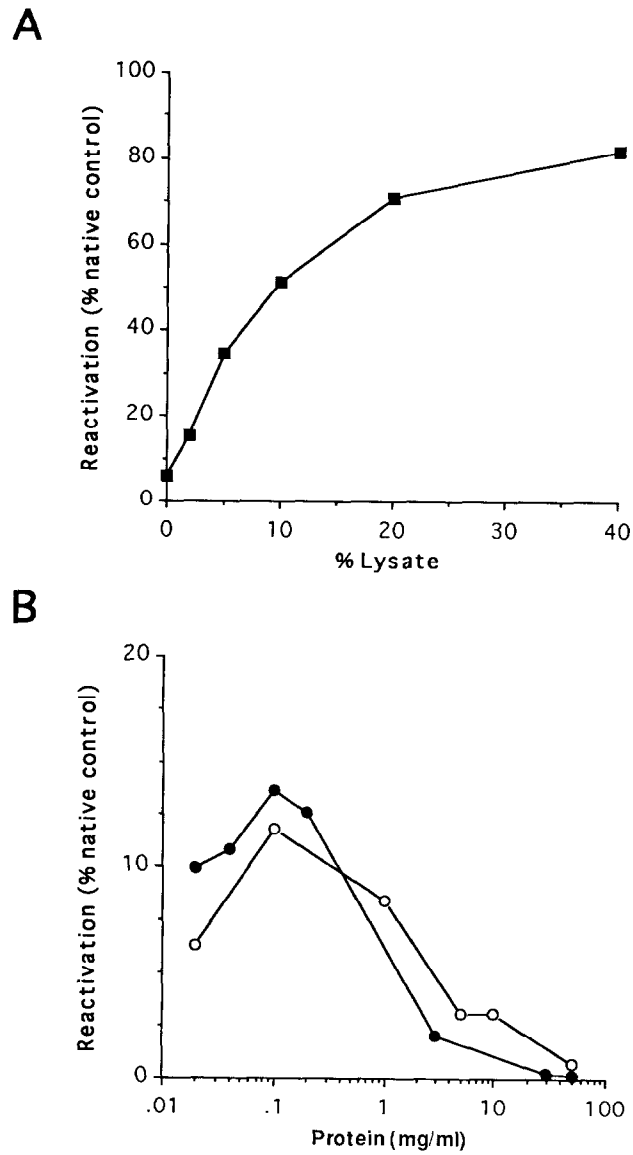


Fig. 1. Dependence of luciferase refolding on reticulocyte lysate. Denatured firefly luciferase was diluted to a final concentration of 0.032 μM into refolding assays containing buffer C, 1 mM ATP and varying amounts of reticulocyte lysate (A), or BSA (○) or haemoglobin (●) (B). Luciferase activities reached after 40 min incubation at 30°C are expressed as percent of the native enzyme control (see section 2).

reticulocyte lysate by gel filtration. This treatment abolished the refolding activity seen with untreated lysate almost completely (Fig. 2). Readdition of both ATP and Mg^{2+} ions was necessary to fully restore refolding, which occurred with an apparent half-time of ~8 min at 30°C. Likewise, chelating of divalent cations by adding EDTA to non-desalted lysate inhibited the refolding of luciferase (not shown). These data indicated that the protein folding activity detected in the reticulocyte cytosol depends on the presence of hydrolyzable ATP.

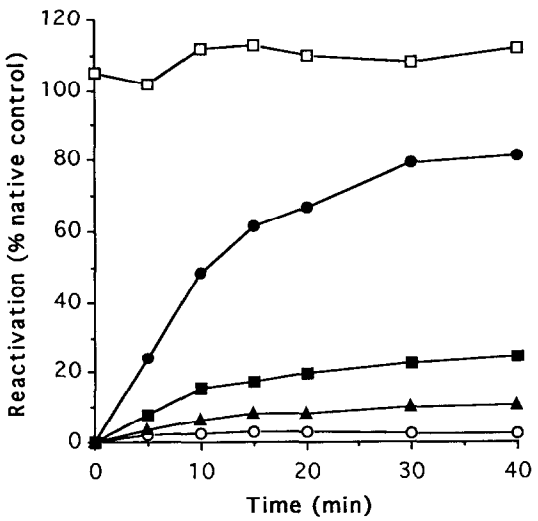


Fig. 2. Mg-ATP-dependence of luciferase refolding. Reactivation of denatured luciferase diluted into buffer B containing 40% desalted lysate was determined either without further addition (○), or with addition of 5 mM $Mg(OAc)_2$ (▲), 1 mM ATP (■) or $Mg(OAc)_2$ and ATP (●). Native luciferase was diluted into buffer B containing 40% desalted lysate and MgATP (□) Activities are expressed as percent of the native enzyme control.

3.2. *Prevention of luciferase aggregation and maintenance of folding competence*

The physical state of luciferase upon dilution of the protein from denaturant was analyzed. When added into refolding buffer at 25°C, the denatured protein aggregated rapidly and was found in the pellet fraction after centrifugation at 16,000 × *g* (Fig. 3a). In contrast, when the protein was diluted into refolding buffer containing 20% desalted reticulocyte lysate, the formation of sedimentable aggregates was prevented. The luciferase protein was then stabilized in the soluble supernatant fraction for at least 30 min. Notably, this was not observed when either BSA or haemoglobin were used to substitute for reticulocyte lysate (not shown). About 70% of luciferase activity was recovered when Mg-ATP was added 1 min after dilution of the protein from denaturant (Fig. 3b). The yield of active enzyme was still about 40%, when refolding was initiated by Mg-ATP after a 30 min incubation at 25°C. We assume that this slow loss of refolding competence, which was not observed upon incubation at 4°C, is due to the limited stability of luciferase–chaperone complexes in the ATP-depleted lysate. In contrast, when denatured luciferase was first mixed with buffer in the absence of reticulocyte lysate, its competence for refolding by the subsequent addition of lysate and Mg-ATP decreased much more rapidly at a rate similar to that of luciferase aggregation (Fig. 3b). In comparing these rates it has to be taken into account that aggregation continues, albeit more slowly, during the 10 min centrifugation at 4°C (see legend to Fig. 3a). Thus, while it cannot be ruled out that reticulocyte lysate may have a limited capacity to resolve lower order aggregates of luciferase, which are not sedimentable at 16,000 × *g*, the predominant reaction measured in our refolding assay is apparently the reactivation of luciferase from a non-aggregated, chaperone-stabilized form.

3.3. *Gel filtration analysis of folding-competent luciferase in reticulocyte lysate*

The capacity of the reticulocyte lysate to maintain the unfolded protein in a folding competent state was further investigated with the aim to eventually identify the components that mediate refolding. GdmCl-denatured

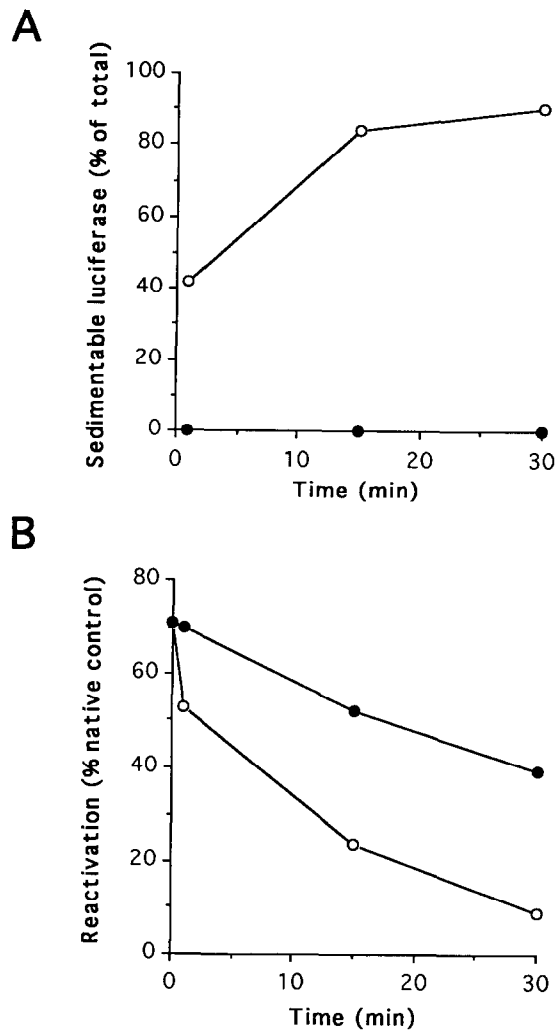
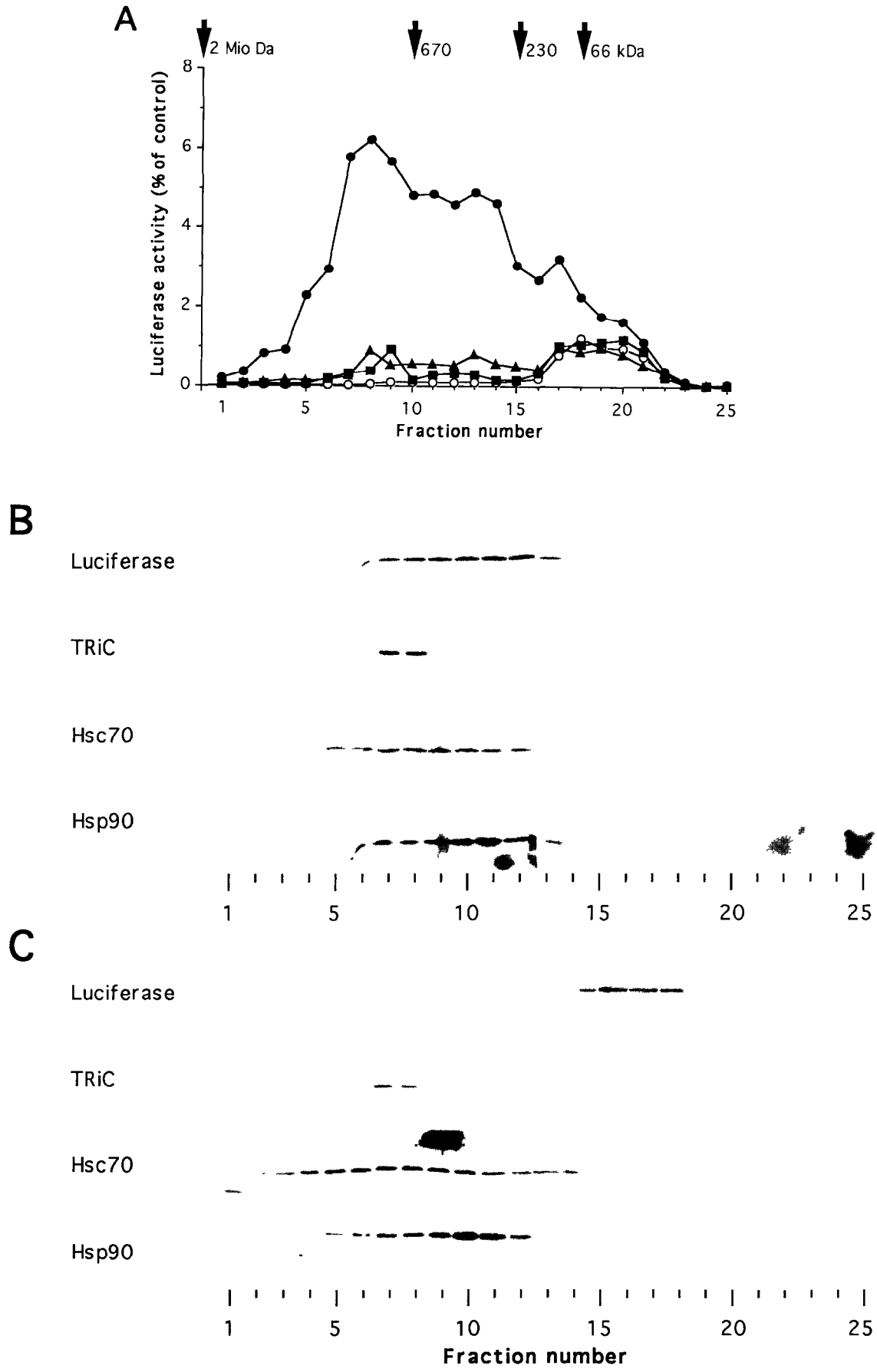


Fig. 3. Maintenance of folding competence of unfolded luciferase. (A) Denatured luciferase (0.2 mg/ml) was diluted into buffer C containing 1 mM ATP (○) or into buffer C containing 20% desalted lysate (●). At the indicated times, the reactions were centrifuged at 16,000 × *g*, 4°C for 10 min. Luciferase in pellet fractions was analyzed by SDS-PAGE and immunoblotting. Sedimentable luciferase is expressed as percent of total luciferase present in the reaction. (B) At the indicated times following dilution of denatured luciferase into buffer C/1 mM ATP (○) or into buffer C/20% desalted lysate (●), refolding was initiated by adding 20% lysate (○) or 1 mM ATP (●), respectively. Activities reached after 40 min incubation at 30°C are expressed as percent of the native enzyme control.


A
2 Mio Da
670
230
66 kDa
Luciferase activity (% of control)
Fraction number
B
Luciferase
TRiC
Hsc70
Hsp90
C
Luciferase
TRiC
Hsc70
Hsp90
Fraction number

luciferase was diluted into desalted reticulocyte lysate and incubated for 15 min at 25°C to ensure that most protein not bound to chaperone had aggregated. Luciferase aggregates were then sedimented and the supernatant of the reaction containing stabilized protein was fractionated on a Superose 6 column (Fig. 4a). About 90% of the luciferase protein analyzed was recovered from the column in a broad distribution between ~1,000 and 250 kDa (Fig. 4b). Only little luciferase was detected at around 60 kDa corresponding to the position of the native enzyme (Fig. 4c). The luciferase in the high molecular weight column fractions 5–15 had almost no enzymatic activity (Fig. 4a). We tested whether this luciferase was competent to undergo ATP-dependent refolding. Surprisingly, addition of Mg-ATP to each column fraction followed by an incubation for 30 min at 30°C was without effect. A similar result was obtained when desalted reticulocyte lysate was added at a concentration comparable to that used in the complete refolding reaction. Only when the column fractions were incubated with desalted lysate *and* Mg-ATP was the efficient regain of luciferase activity observed. The final yield in activity over all column fractions was about 65% of that obtained in the unfractionated lysate. Peak activities were reached with luciferase that fractionated at ~1,000 kDa (fractions 7 and 8), although these fractions contained less luciferase protein than fractions 11 and 12, for example. These results indicate that distinct activities, presumably molecular chaperone proteins of different classes and their cofactors, cooperate in protein refolding in the reticulocyte lysate.

The fractionation properties of the major known molecular chaperone proteins in the cytosol, namely Hsc70, Hsp90 and the chaperonin TRiC, were analyzed in the presence or absence of denatured luciferase (Fig. 4b and c). While the distribution of Hsc70 largely overlapped with the high molecular weight complexes of luciferase, this separation behaviour was apparently independent of the presence of the denatured protein. Similar observations were made with Hsp90. It has to be considered that a large amount of the total Hsc70 and Hsp90 may already be bound to polypeptide substrates naturally present in the reticulocyte lysate. In the case of TRiC, a shift in fractionation upon luciferase binding is not expected due to the high molecular weight of this chaperonin (~970 kDa) [13]. Interestingly, the peak reactivation of luciferase was measured in the TRiC containing fractions (Fig. 4a). The participation of TRiC in luciferase folding is indeed likely, based on our previous observation that purified bovine TRiC can support the refolding of this protein in vitro [13].

4. DISCUSSION

We have described a simple and reliable assay to measure the refolding activity of a eukaryotic cytosolic extract. Firefly luciferase was chosen as the substrate because: (i) its spontaneous refolding upon dilution from denaturant is inefficient; (ii) the luminescence based enzyme assay of luciferase is extremely sensitive, thus allowing to test refolding activities in small fraction volumes; (iii) the enzyme assay is very fast so that any reactivation during the measurement is minimal. We believe that the luciferase renaturation assay will be useful in identifying the molecular chaperone components functioning in protein folding in various cell types and cellular compartments.

Analyzing the renaturation of luciferase, the cytosol of rabbit reticulocytes was found to contain a highly efficient ATP-dependent protein folding activity. In the absence of hydrolyzable ATP, the aggregation of luciferase added to the lysate from 6 M GdmCl was prevented. The protein remained in a stable, folding competent conformation from which it could reach the native state upon subsequent addition of ATP. The possibility to distinguish between a binding step and refolding allowed the chromatographic fractionation of the reticulocyte lysate containing stabilized luciferase. These experiments revealed an unexpected level of complexity for cytosolic protein folding. While more than 90% of luciferase protein was recovered from the sizing column, additional components were required to obtain ATP-dependent reactivation. Either specific cofactors were removed from the chaperone-stabilized luciferase during fractionation and/or different chaperone activities have to cooperate in luciferase refolding. Possible components participating in this process are Hsc70 and Hsp90 with their respective cofactors, as well as the recently discovered chaperonin TRiC (for recent reviews see [1,2]).

A functional synergy of *E. coli* Hsp70 (DnaK) and the chaperonin GroEL in protein folding [5] and prevention of aggregation [6] has recently been described. This reaction depends on the chaperone cofactors DnaJ, GrpE

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Fig. 4. Fractionation by gel filtration of folding-competent luciferase in desalted reticulocyte lysate. (A) Gel filtration of the reticulocyte lysate reaction was performed as described in section 2. Luciferase activity was determined immediately after the column fractionation (○), or after a 30 min incubation at 30°C with either 1 mM ATP (■), 50% desalted lysate (▲) or 1 mM ATP and 50% desalted lysate (●). Activities per fraction are expressed as percent of the total activity reached in the unfractionated refolding reaction after 30 min incubation in the presence of Mg-ATP. The peak fractions of the following molecular weight standards are indicated: Blue dextran, 2×10^6 Da; thyroglobulin, 670 kDa; catalase, 230 kDa; BSA, 66 kDa. (B) The fractionation pattern of luciferase and the chaperone proteins TRiC, Hsc70 and Hsp90 was analyzed by SDS-PAGE and immunoblotting with specific antibodies (see section 2). (C) Native luciferase (10 μM) was diluted 100-fold into desalted reticulocyte lysate and the reaction fractionated on a Superose 6 column as in A. The fractionation pattern of the indicated proteins was determined as in B.

and GroES. A similar pathway of chaperone-mediated protein folding may be operative in the reticulocyte lysate. While DnaJ homologues in the eukaryotic cytosol have already been identified, a GrpE-like component has not yet been discovered in this compartment [21,22]. Based on preliminary results, non-native luciferase in the high molecular weight column fractions appears to be associated with TRiC (E.N. and F.U.H., unpublished observations). The requirement of complete reticulocyte lysate for refolding in these fractions in addition to ATP would then suggest that TRiC functions together with an as yet unidentified factor(s). Hsc70 and Hsp90 are unlikely to be limiting, giving their abundance in the TRiC containing fractions. Interestingly, a cofactor dependence has not been observed for the refolding of denatured luciferase by purified bovine TRiC [13]. However, two distinct additional factors are apparently required for the folding/assembly of tubulin by rabbit reticulocyte TRiC [23]. A detailed analysis of the reticulocyte lysate system based on the sensitive luciferase refolding assay should provide important insight into the pathways of assisted protein folding in the eukaryotic cytosol.

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REFERENCES

[1] Gething, M.-J. and Sambrook, J. (1992) Nature 355, 33–45
[2] Hendrick, J.P. and Hartl, F.U. (1993) Annu. Rev. Biochem. 62, 349–384.
[3] Pfanner, N. and Neupert, W. (1990) Annu. Rev. Biochem. 59, 331–353.
[4] Hartl, F.U., Martin, J. and Neupert, W. (1992) Annu. Rev Biophys. Biomol. Struct. 21, 293–322.
[5] Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M.K. and Hartl, F.U (1992) Nature 356, 683–689.
[6] Gragerov, A., Nudler, E., Komissarova, N., Gaitanaris, G.A., Gottesman, M.E and Nikiforov, V. (1992) Proc. Natl. Acad Sci. USA 89, 10341–10344
[7] Beckmann, R.P., Mizzen, L.A. and Welch, W.J. (1990) Science 248, 850–854.
[8] Nelson, R.J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M. and Craig, E A. (1992) Cell 71, 97–105.
[9] Wiech, H., Buchner, J., Zimmermann, R and Jacob, U. (1992) Nature 357, 200–205.
[10] Gao, Y., Thomas, O , Chow, R.L., Lee, G.-H. and Cowan, N.J. (1992) Cell 69, 1043–1050.
[11] Yaffe, M B., Farr, G.W., Miklos, D., Horwich, A.L., Sternlicht, M.L. and Sternlicht, H. (1992) Nature 358, 245–248.
[12] Lewis, V.A., Heynes, G M , Zheng, D., Saibil, H and Willison, K. (1992) Nature 258, 249–252.
[13] Frydman, J., Nimmesgern, E., Erdjument-Bromage, H., Wall, J S., Tempst, P. and Hartl, F.U. (1992) EMBO J. 11, 4767–4778
[14] Keller, G.-A., Gould, S., DeLuca, M. and Subramani, S. (1987) Proc. Natl Acad. Sci. USA 84, 3264–3268.
[15] Schroder, H., Langer, T , Hartl, F.U. and Bukau, B. (1993) EMBO J., in press.
[16] Laemmli, U K. (1970) Nature 227, 680–685.
[17] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 79, 267–271.
[18] Willison, K., Lewis, V., Zuckerman, K.S., Cordell, J., Dean, C., Miller, K., Lyon, M.F. and Marsh, M. (1989) Cell 57, 621–632.
[19] Vachereau, A. (1989) Anal. Biochem. 179, 206–208.
[20] deWet, J R., Wood, K.V., DeLuca, M , Helinski, D.R. and Subramani, S. (1987) Mol Cell. Biol. 7, 725–737.
[21] Georgopoulos, C. (1992) Trends Biochem Sci 17, 295–299
[22] Caplan, A J., Cyr, D.M. and Douglas, M.G (1993) Mol Biol Cell 4, 555–563.
[23] Gao, Y., Vainberg, I E , Chow, R.L. and Cowan, N J. (1993) Mol. Cell. Biol. 13, 2478–2485.