Enzyme Assembly after de Novo Synthesis in Rabbit Reticulocyte Lysate Involves Molecular Chaperones and Immunophilins* 

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The folding kinetics of two luciferases were studied after synthesis in reticulocyte lysates to investigate whether molecular chaperones and/or folding catalysts are involved in the folding reactions. Two bacterial luciferases were used as model proteins: heterodimeric Vibrio harveyi luciferase (LuxAB), and a monomeric luciferase fusion protein (Fab2). Data indicate that folding of these enzymes to the native state occurs in the translation system, and that the extent of folding can be quantified. It was found that (i) folding of LuxAB and Fab2 can clearly be separated in time from synthesis, (ii) folding of Fab2 and LuxAB is slow because it involves either transient (Fab2) or permanent (LuxAB) interaction of polyepitides, (iii) preservation of the assembly competent state of LuxA and/or LuxB and folding of Fab2 depend on ATP-hydrolysis, (iv) folding of Fab2 and LuxAB is partially sensitive to cyclosporin A (CsA) and FK506, i.e. inhibitors of two distinct peptidylprolyl cis/trans-isomerases. Thus, bacterial luciferases provide a unique system for direct measurement of the effects of ATP-dependent molecular chaperones on protein folding and enzyme assembly in reticulocyte lysates. Furthermore, these two luciferases provide the first direct evidence documenting the involvement of peptidylprolyl cis/trans-isomerases in protein biogenesis in an eukaryotic cytosol.

Although much is known about the rules and mechanisms governing protein folding after denaturation and subsequent renaturation (Jaenicke, 1987), in contrast little is known about protein folding and subunit assembly following de novo synthesis of polyepitides. The latter is generally assumed to be assisted by molecular chaperones and folding catalysts that are present in all organisms and in all cellular compartments (Ellis and van der Vies, 1991; Gething and Sambrook, 1992; Georgopoulos and Welch, 1993; Hartl et al., 1994; Schreiber, 1991). Chaperones, such as the two ATP-dependent classes of chaperones, the heat shock protein (Hsp) families Hsp60 and Hsp70, are believed to prevent aggregation of unfolded polyepitides by stabilizing folding intermediates. Folding catalysts, such as peptidylprolyl cis/trans-isomerases (PPlases), are supposed to increase the rate of slow folding steps. The PPlases do also function as intracellular receptors for immunosuppressants and are therefore also named immunophilins (Schreiber, 1991). The PPlases include two structurally distinct protein families with different, but overlapping substrate specificities: the cyclophilins have high affinity binding sites for the drug cyclosporin A (CsA), while the FK506-binding proteins (FKBPs) bind FK506. In both cases, binding of the drug inhibits isomerase activity of the enzyme. FK506 inhibits the PPlase activity of FKBPs, but not of cyclophilins; likewise, CsA does not inhibit the PPlase activity of FKBPs. Here we directly addressed questions related to protein folding by employing cell-free translation systems, such as rabbit reticulocyte lysates. Specifically, we asked (i) what are the kinetics of folding and assembly of light emitting model proteins in the reticulocyte lysate, and (ii) are ATP-dependent molecular chaperones and/or folding catalysts involved in the folding reactions.

In order to be able to follow the folding kinetics of a certain protein in a reasonable amount of translation reaction (where about 10–100 fmol/μl of protein is synthesized), enzymes catalyzing light emitting reactions were chosen as model proteins. In the experiments described below two bacterial luciferases were used. The first luciferase (LuxAB) is a heterodimeric enzyme from Vibrio harveyi MAV (Waddell et al., 1987; Escher et al., 1989; Flynn et al., 1993; Ziegler et al., 1993). The second luciferase is a fusion protein (Fab2) which forms a monomeric enzyme comprising LuxA and LuxB harboring a 10-amino acid residue containing linker peptide between the carboxyl terminus of the α-subunit and the amino terminus of the β-subunit (Escher et al., 1989). Both luciferases catalyze the oxygen- and FMN-dependent conversion of a long chain aldehyde to the corresponding fatty acid with concomitant emission of light. In the case of the heterodimer the two subunits were observed to fold cooperatively after expression in Escherichia coli and after denaturation and subsequent renaturation (Waddell et al., 1987). Furthermore, it was shown that after separate expression of the α- and β-subunits in E. coli the subunits can be stabilized in an assembly competent state by the bacterial molecular chaperone GroEL (Flynn et al., 1993). In addition it was observed that upon denaturation/renaturation there are slow steps in the refolding of luciferase subunits and in the forma-

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1 The abbreviations used are: Hsp, heat shock protein; PPlase, peptidylprolyl cis/trans-isomerase; CsA, cyclosporin A; ATP-S, adenosine 5’-O-thiotriphosphate; FKB, FK506-binding protein; TrIC, complex polypeptide ring complex.
RESULTS

*Folding of Bacterial Luciferases at Various Temperatures*—The genes coding for LuxA, LuxB, and Fab2 were cloned into plasmids suitable for *in vitro* transcription. The plasmids were transcribed and the transcripts subsequently used to program translation in rabbit reticulocyte lysates. In order to follow the kinetics of synthesis of the respective polypeptide chains, the translation reaction was carried out in the presence of $^{[35S]}$methionine, aliquots of the translation reactions were taken at different times and followed by gel electrophoresis and fluorography analyses. In order to follow the kinetics of folding of the different enzymes, the translation was carried out in the absence of a labeled amino acid and aliquots were taken and analyzed in the presence of the required substrates of the luciferase reaction by luminometry.

Originally, these studies were carried out under conditions of coexpression of LuxA and LuxB (resulting in similar concentrations of LuxA and LuxB proteins) and at defined temperatures between 23 and 30 °C. In a first set of experiments we observed that the rate of LuxAB and Fab2 folding was slow when compared to the rate of protein synthesis (data not shown). Therefore, in subsequent experiments protein synthesis was inhibited by the addition of cycloheximide and RNase A at an appropriate time of translation. The efficiency of this translation arrest has been previously demonstrated (Wiech et al., 1987) and is shown in Fig. 1. A and B. LuxA plus LuxB proteins and Fab2, respectively, were synthesized at 23, 25, 28, and 30 °C. After 90 or 120 min the translation reaction was inhibited and the incubation for folding was continued at the same temperature. As expected, the yields of the newly synthesized polypeptides increased with increasing temperature until the inhibitors of translation were added (Fig. 1. A and B). Accordingly, the yield of luciferase activity increased with increasing levels of expression in the case of the heterodimeric enzyme (Fig. 1C). However, in the case of the fusion protein the highest yield of luciferase activity was detected after translation and a incubation for folding at 25 °C (Fig. 1D). Taking into account the different expression levels of Fab2 at the various temperatures, i.e. calculating specific enzyme activities (Fig. 1, E and F), it appeared that Fab2 showed an identical temperature-sensitive phenotype for folding which had previously been observed after expression in *E. coli* (Escher et al., 1989). Thus, optimal specific activity of the enzyme was observed at 25 °C and decreasing levels were detected at increasing temperatures (Fig. 1F). Both luciferases showed slower folding reactions when compared to the rate of translation. At 23 and 25 °C the folding of the heterodimeric as well as that of the monomeric luciferase could almost be completely separated in time from the synthesis of the various polypeptides (Fig. 1, C and D). We note that after synthesis at 23 °C expression efficiency and folding yield were similar for LuxAB and Fab2 (Fig. 1, E and F). When the translation reactions were shifted to 0 °C after inhibition of protein synthesis, no increase in the enzyme activities occurred (data not shown).

Therefore, the two bacterial luciferases provide a unique system for studying folding and enzyme assembly in a cell-free translation system, thus eliminating the need for the use of amino acid analogs or the use of truncated polypeptide chains.

*Folding of Bacterial Luciferases Is Sensitive to Dilution*—In order to demonstrate the bimolecular nature of LuxAB folding and the monomolecular nature of Fab2 folding, respectively, and in order to ask whether components of the reticulocyte lysate can become limiting for the respective folding reaction, the kinetics of folding of the different enzymes were studied after dilution. In these experiments the translation reactions were supplemented either with rabbit reticulocyte lysate (*i.e.*
FIG. 1. Kinetics for expression and folding of LuxAB and Fab2 at different temperatures. Plasmids pLX203ab, pLX203-b, and pLX709ab2 were separately transcribed with T7 polymerase. Translation in reticulocyte lysates in the presence of transcripts, coding for LuxA and LuxB (A, C, and E) or Fab2 (B, D, and F), plus [35S]methionine (A and B) or plus unlabeled methionine (C and D) was carried out at 23, 25, 28, and 30 °C, as indicated. After 90 and 120 min (indicated by arrow), respectively, cycloheximide (final concentration: 100 µg/ml) and RNase A (final concentration: 80 µg/ml) were added to stop the translation reaction and the incubation was allowed to proceed. At the indicated times aliquots were withdrawn and analyzed for luciferase content (A and B) and activity (C and D), respectively. In panels A-D data for three temperatures are shown. The apparent specific activity, shown in E and F, represents the enzyme activity which was determined at a certain time of the folding reaction (relative light units (rlu) x 10^{-5}), divided by the amount of completed polypeptide chains which were present at the time of translation stop (arbitrary units).

keeping the concentrations of reticulocyte lysate proteins constant) or with buffer (experiments where the concentration of the lysate proteins was lowered).

LuxA plus LuxB and Fab2, respectively, were synthesized at 23 °C. After 90 or 120 min, the translation reaction was terminated and the translation mixtures were divided into three aliquots. The first aliquot was left undiluted, the second aliquot was diluted 2-fold with the same reticulocyte lysate, and the third aliquot was diluted 2-fold with buffer. Then, the incubation was allowed to continue at 23 °C and the luciferase enzyme activities were monitored. Folding of LuxAB (Fig. 2A) and Fab2 (Fig. 2B) was equally sensitive to dilutions under the two conditions. If similar dilutions were made after completion of folding reactions but prior to the activity measurements, dilutions had no effect on enzyme activity (data not shown). Thus, it appears that both folding of LuxAB and Fab2 polypeptides in

the reticulocyte lysate are bimolecular reactions which involve either transient (Fab2) or permanent (LuxAB) interaction of two translation products. The finding that coexpression of either luxA or luxB gene products together with Fab2 led to an increase in the yield of enzymatically active Fab2, regardless of its synthesis in E. coli cells (Escher et al., 1989) or in reticulocyte lysate (data not shown) further supports the interpretation that the fusion protein is able to interact with another protein during its folding. It seems that folding of both luciferases is slow because even in the undiluted translation reactions both the concentrations of the various polypeptides and the chances for two polypeptides to interact are low and limiting for folding. Therefore, the folding kinetics were slower under conditions of low expression levels, such as was observed at 23 °C (see above). Due to the pronounced sensitivities of the folding reactions to dilution in reticulocyte lysate, we were unable to detect that reticulocyte lysate proteins limited the folding of LuxAB or Fab2 under these experimental conditions.

**Folding of Bacterial Luciferases Involves ATP-hydrolysis**—To determine the involvement of molecular chaperones, the kinetics of folding of the different enzymes were studied after supplementing the translation mixture with inhibitors of ATP-dependent molecular chaperones. First the folding reactions were analyzed to determine their sensitivities to ATP depletion. In a second and third set of experiments we asked whether the addition of ATP or nonhydrolyzable analogs of ATP, such as ATPγS, prevent the effect of ATP depletion or whether ATPγS interferes with the folding reactions.

In these experiments the two subunits of the heterodimeric enzyme were synthesized separately, subsequently subjected to the various treatments, then combined (resulting in similar concentrations of LuxA and LuxB) and incubated further. LuxA, LuxB, and Fab2, respectively, were synthesized at 23 °C. The translation was allowed to proceed for 35 or 60 min, and then the translation mixtures were divided into several aliquots. In the first experiment, the first aliquot was left untreated and the second aliquot was supplemented with apyrase, an enzyme which catalyzes the hydrolysis of ATP and ADP. The third aliquot was supplemented with increased amounts of apyrase (Fig. 3, A and B). The incubation was continued at 23 °C and the enzyme activities were monitored.
under these various conditions. Folding of LuxA (Fig. 3A) and Fab2 (Fig. 3B) was similarly sensitive to appyrase treatment. The inhibitory effect of appyrase was dependent on the appyrase concentration (Fig. 3, A and B) and reached a maximum at around 30 (LuxAB) and 55% (Fab2) inhibition under these conditions (Fig. 4). In a follow-up experiment, the first aliquot was left untreated, the second aliquot was supplemented with appyrase, the third aliquot was supplemented with appyrase plus ATP, and the fourth aliquot was supplemented with appyrase plus ATP·S (Fig. 3, C and D). The effect of appyrase was due to depletion of ATP since (i) denatured appyrase had no detectable inhibitory effect (data not shown), and (ii) the appyrase effect was prevented by ATP but not by ATP·S (Fig. 3, C and D). In a third experiment, the first aliquot was left untreated, the second aliquot was supplemented with ATP, and the third aliquot was supplemented with ATP·S (Fig. 3, E and F). It was found that ATP·S competed with ATP and had an inhibitory effect on folding of LuxA and Fab2, similar to that of appyrase (Fig. 3, E and F). When appyrase was added after completion of LuxA or Fab2 folding it had no effect on the enzyme activity (data not shown). Therefore, these experiments strongly suggest that ATP depletion does not interfere with the catalytic activity of the two enzymes, but affects the folding reaction.

We conclude from this set of data that folding of the heterodimeric luciferase occurs after separate synthesis of the two subunits in reticulocyte lysates. Under these conditions the assembly-competent subunits lose their competence in a time-dependent fashion, and no enzymatically active homodimers are observed when the separately synthesized subunits are separately incubated further (data not shown). Furthermore, on the basis of these results we conclude that folding of both the monomeric and the heterodimeric luciferases involves the hydrolysis of ATP and, therefore, the folding is drastically reduced either by ATP depletion or by competition with ATP·S. We note that these inhibitory effects are found to be most pronounced when the inhibitor is added after short translation (Fig. 4) and that under all conditions they can be prevented by the addition of ATP (data not shown). Furthermore, we note that the effect of ATP depletion or ATP·S after coexpression of LuxA and LuxB is much less pronounced when compared to the separate expression experiments (data not shown), suggesting that the ATP-dependent step occurs early in the folding of the two enzymes, i.e., prior to the assembly reaction.

**Fig. 4.** Folding of LuxAB and Fab2 is ATP-dependent during early stages of folding. LuxA, LuxB, and Fab2 were synthesized for a given time, supplemented with water or appyrase (20 and 2 units/ml, respectively), and incubated further as described in the legend to Fig. 3. The translation time was varied between 45 and 65 min for LuxA/LuxB and between 28 and 60 min for Fab2. The inhibitory effect of ATP depletion on LuxAB or Fab2 folding was determined and was plotted against the translation time (i.e., the time of cycloheximide and RNase A addition).
incubation was allowed to continue at 23 °C and the enzyme activities were measured. In addition, the two subunits of the heterodimeric enzyme were also synthesized separately, combined subsequently (resulting in similar concentrations of LuxA and LuxB), and incubated further under the three conditions described above. Folding of LuxAB under these latter conditions (Fig. 5, A and C) and Fab2 (Fig. 5, B and D) were partially sensitive to each of the two drugs. The effect of the drugs after coexpression of LuxA and LuxB was similar (Fig. 5F). The dose-response curve which was generated for LuxAB under these conditions showed a half-maximal effect of the two inhibitors at concentrations between 5 and 10 μg/ml (i.e. 5–10 μM) and reached a plateau of approximately 60% inhibition at 40 μg/ml (CaA) and 100 μg/ml (FK506), respectively (Fig. 5F). When addition of CaA or FK506 occurred after completion of folding of LuxAB or Fab2 in the untreated samples, there was no effect on the yield of enzyme activity (data not shown). Thus, the two drugs did not interfere with the catalytic activity of the two enzymes, but rather affected their folding. We concluded from this set of data that proline isomerization may be involved in folding of LuxAB and Fab2 and that the amount of assembly-competent polypeptides is partially reduced by inhibition of either CaA- or FK506-sensitive PPIases which are undoubtedly present in the reticulocyte lysate (see “Experimental Procedures”) since they have been found in various other blood cells (Foxwell et al., 1988; Schreiber, 1991).

We therefore reasoned that simultaneous inhibition of both types of PPIases should result in a more pronounced inhibition of luciferase folding. Thus the folding kinetics of the heterodimeric enzyme were studied after supplementing the translation mixture with the two inhibitors at the same time. LuxA and LuxB were synthesized at 23 °C. After the translation reaction was inhibited, the combined translation mixture (containing similar concentrations of LuxA and LuxB) was divided into four aliquots. The first aliquot was supplemented with MesSO, the second and third aliquots were supplemented with, respectively, CaA and FK506, and the fourth aliquot was supplemented with CaA plus FK506. Then the incubation was allowed to continue at 23 °C and the enzyme activities were measured. Folding of LuxAB under these conditions (Fig. 5E) was found to be more sensitive to a combined addition of CaA and FK506 than to the addition of CaA or FK506 alone, therefore corroborating a possible role of PPIases in the folding of newly synthesized luciferases.

In order to demonstrate the inhibitory effect of CaA and FK506 on PPIases directly, the translation mixture was supplemented with purified cyclophilin (i.e. a CaA-sensitive PPIase) and the folding kinetics of the heterodimeric enzyme were studied in the absence or presence of FK506. The two subunits of the heterodimeric enzyme were synthesized separately, combined subsequently (resulting in similar concentrations of LuxA and LuxB), and incubated further under various conditions. LuxA and LuxB were synthesized at 23 °C. After inhibition of the translation process, the combined translation mixture was divided into four aliquots. The first aliquot was supplemented with MesSO, the second aliquot was supplemented with MesSO plus human cyclophilin, the third aliquot was supplemented with FK506, and the fourth aliquot was supplemented with FK506 plus cyclophilin. The incubation was allowed to continue at 23 °C and the enzyme activities were monitored by luminometry. After analyzing these data we found that the inhibitory effect of FK506 on luciferase folding was partially suppressed by addition of cyclophilin (Fig. 6, A and B). In a follow-up experiment we asked whether the ability of cyclophilin to suppress the inhibitory effect of FK506 depends on the native state of the enzyme (Fig. 6B). Both, heat
and CsA pretreatment of cyclophilin inhibited its stimulatory effect on luciferase folding in the presence of FK506.

Therefore, we conclude that at least a significant proportion of the inhibitory effect of FK506 on luciferase folding is due to an inhibition of FKBP5s which are present in the reticulocyte lysate and not due to either direct or indirect effects of this drug on LuxA and/or LuxB. We also suggest that the same is true for cyclosporin A. Furthermore, we conclude that proline isomerization may indeed be involved in folding of LuxAB and that PPIases which are present in reticulocyte lysates may become limiting under conditions of partial inhibition of PPIase activity. On the basis of these results we propose that one of the slow folding steps which was observed for the folding of the bacterial luciferase in denaturation/renaturation studies (Ziegler et al., 1993) is related to proline isomerization. However, it is possible that luciferase folding involved the general chaperoning activity of the immunophilins (Freskgard et al., 1992) rather than their PPIase activity. In any case, sensitivity of luciferase folding to CsA and FK506 and the partial suppression of the inhibitory effect of FK506 by the addition of cyclophilin provide the first direct evidence for the involvement of immunophilins in protein biogenesis in a eukaryotic cytosolic system.

**DISCUSSION**

Relatively little is known about protein folding and subunit assembly following de novo synthesis of polypeptides in the eukaryotic cytosol (Ellis and van der Vies, 1991; Gething and Sambrook, 1992; Georgopoulos and Welch, 1993; Hartl et al., 1994). The best studied example for the latter folding conditions is the folding of β-tubulin and its subsequent assembly with pre-existent α-tubulin (Yaffe et al., 1992). Here, we describe studies on protein folding using two enzymes catalyzing light emitting reactions, by employing rabbit reticulocyte lysate as an environment similar to the eukaryotic cytosol. The two bacterial luciferases, LuxAB and Fab2, were chosen as model enzymes because their enzymatic activity is easily detected (high sensitivity, no background) and due to the available information about their folding characteristics after denaturation followed by renaturation in vitro as well as after their expression in E. coli cells (Waddell et al., 1987; Olsson et al., 1988; Escher et al., 1989; Flynn et al., 1993; Ziegler et al., 1993; Escher and Szalay, 1993). Reticulocyte lysate appeared to us as the eukaryotic cytosol of choice since it allows high yields in expression. Furthermore, it has been shown in denaturation/renaturation studies that the system contains various molecular chaperones (such as t-complex polypeptide 1 ring complex TrIC, and Hsc70) (Gao et al., 1992; Rommelaere et al., 1993; Nimmesgern and Hartl, 1993; Schumacher et al., 1994).

Here, we demonstrate that folding and assembly of the two luciferases does occur after synthesis in the cell-free translation system. We also show that the extent of folding and assembly can be quantified under these conditions. Furthermore, bacterial luciferases proved to be a unique system for direct demonstration of the effects of molecular chaperones in protein biogenesis. The following folding characteristics were observed for the two enzymes. (i) Folding of LuxAB and Fab2 can clearly be separated in time from synthesis at temperatures of 23 and 25 °C. (ii) Folding of LuxAB and Fab2 shows cooperativity. (iii) Following separate synthesis, the two subunits of the heterodimeric enzyme stay assembly competent for a limited time. (iv) Preservation of the assembly competent state of LuxA and/or LuxB and folding of Fab2 are dependent on the hydrolysis of ATP. Therefore, it appears that folding of these luciferases involves ATP-dependent molecular chaperones. The most likely explanation for the observed ATP effect is that TrIC may be involved in the folding of the two luciferases in the reticulocyte lysate. This was demonstrated earlier to be the case in β-tubulin folding (Yaffe et al., 1992). Furthermore, the bacterial homolog of TrIC, GroEL, was previously shown to be able to preserve the assembly competent state of the two subunits of the heterodimeric enzyme after separate expression in E. coli cells and (in collaboration with GroES) to reverse the temperature-sensitive folding phenotype of the hybrid luciferase (Escher and Szalay, 1993; Flynn et al., 1993). At this time Hsc70 cannot be excluded as an alternative or additional contributor to the ATP-dependent folding. Hsc70 is assumed to be associated with newly synthesized polypeptide chains (Beckman et al., 1990; Nelson et al., 1992) and has been observed to play a role in protein topogenesis in an ATP-dependent manner (Chirico et al., 1988; Deshaies et al., 1988; Zimmermann et al., 1988).

PPIases catalyze conversion between cis- and trans-isomers of proline-containing amide bonds of peptides and proteins in vitro. PPIases are abundant proteins and belong to either one of two related protein families which can be distinguished by their sensitivity to immunosuppressant drugs, such as CsA and FK506 (Lang et al., 1987; Takahashi et al., 1989; Fischer et al., 1989; Tropschug et al., 1989, 1990). However, the cellular functions of the PPIases have not yet been established. Three observations pointed to a role of PPIases in protein folding following de novo synthesis of polypeptides in vitro. CsA inhibits the folding of the triple helix of type I collagen in the endoplasmic reticulum of fibroblasts (Davis et al., 1989; Steinmann et al., 1991). In Drosophila melanogaster the cyclophilin homolog ninA is essential for transport of rhodopsin through the secretory pathway (Colley et al., 1991). In addition, some of the cyclophilins have been shown to be involved in the heat shock response in yeast (Sykes et al., 1993). Here, we asked if the folding of two luciferases after de novo synthesis in rabbit reticulocyte lysates involves endogenous
immunophlin. The following observations were made: (i) folding of Fab2 and LuxAB, respectively, is partially sensitive to CaA and FK506. (ii) A combination of CaA and FK506 leads to almost complete inhibition of folding. (iii) The inhibitory effect of FK506 on folding can be suppressed by exogenously added cyclophilin, i.e. a CaA-sensitive PPIase. Therefore, it appears that folding of both luciferases involves PPIases, cyclophilins as well as FKBP5s presumably present in the reticulocyte lysate. Thus, these two luciferases provide the first direct evidence documenting the involvement of immunophilins in protein biogenesis in an eukaryotic cytosol.

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