Luciferase Assembly after Transport into Mammalian Microsomes Involves Molecular Chaperones and Peptidyl-Prolyl cis/trans-Isomerases*

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The assembly of a heterodimeric luciferase was studied after de novo synthesis of corresponding precursor proteins in reticulocyte lysate and concomitant transport into dog pancreas microsomes. This cytosolic luciferase from a prokaryotic organism (Vibrio harveyi) was specifically used as a model protein to investigate (i) whether the eukaryotic cytosol and the microsomal lumen have similar folding capabilities and (ii) whether the requirements of a polypeptide for certain molecular chaperones and folding catalysts are determined by the polypeptide or the intracellular compartment. The two luciferase subunits were fused to the preprolactin signal peptide. Data indicate that efficient assembly of luciferase occurs in the mammalian microsomes. Furthermore, it was observed that luciferase assembly can be separated in time from synthesis and membrane transport, depends on ATP hydrolysis, is partially sensitive to cyclosporin A and FK506, and in the absence of luminal proteins is less efficient as compared with the presence of luminal proteins. Thus, heterodimeric luciferase depends on functionally related molecular chaperones and folding catalysts during its assembly in either the eukaryotic cytosol or the microsomal lumen.

In comparison to our knowledge about protein folding after denaturation and subsequent renaturation (Jaenicke, 1987), little is known about protein folding and assembly in the different compartments of the eukaryotic cell, following de novo synthesis of polypeptides. In general, the latter appears to be assisted by various molecular chaperones and folding catalysts (Ellis and van der Vies, 1991; Georgopoulos and Welch, 1993; Hartl et al., 1994; Kunz and Hall, 1993; Schreiber, 1991). However, the intracellular compartments are different from each other with respect to their specific set of molecular chaperones and folding catalysts (Gething and Simmbrook, 1992). Recently, we studied the folding and assembly of newly synthesized proteins in the eukaryotic cytosol by employing rabbit reticulocyte lysate as a translation and folding system (Kruse et al., 1995). We asked (i) what are the kinetics of folding and assembly of two model proteins, and (ii) are ATP-dependent molecular chaperones and/or folding catalysts involved in the folding and assembly reactions? In our studies two bacterial luciferases were used as model proteins. The first luciferase was a heterodimeric enzyme (LuxAB) from Vibrio harveyi (Waddle et al., 1987; Escher et al., 1989; Flynn et al., 1993; Ziegler et al., 1993). The second luciferase was a fusion protein (Fab2) that forms a monomeric enzyme comprising LuxA and LuxB (Escher et al., 1989). Both enzymes catalyze the oxygen- and FMNH₂-dependent conversion of a long chain aldehyde to the corresponding fatty acid with concomitant emission of light (490 nm). The genes coding for LuxA, LuxB, and Fab2 were cloned into plasmids that are suitable for in vitro transcription. The plasmids were used to program coupled transcription/translation in rabbit reticulocyte lysates. The kinetics of folding and assembly of the respective enzymes were studied after supplementing the translation mixture with inhibitors of ATP-dependent molecular chaperones and inhibitors of PPIases, respectively. Using this system we were able to demonstrate that folding and assembly of the two luciferases occur in the in vitro translation system and that the extent of folding and assembly can be quantified. Furthermore, we observed that the two bacterial luciferases involve ATP-dependent molecular chaperones and PPIases in their folding and assembly in a mammalian cytosol.

Here we asked the identical questions related to protein folding and assembly in the mammalian endoplasmic reticulum by employing rabbit reticulocyte lysate as a translation system and dog pancreas microsomes as a folding compartment. The genes coding for LuxA and LuxB were fused with a cDNA, coding for the preprolactin signal peptide. The plasmids were used to program coupled transcription/translation in rabbit reticulocyte lysates in the presence of dog pancreas microsomes (i.e. in the presence of luminal proteins) or proteoliposomes (i.e. in the absence of luminal proteins). The kinetics of assembly of the heterodimeric enzyme were studied under various conditions. We observed that bacterial luciferase involves ATP-dependent molecular chaperones and folding catalysts in its folding and assembly in mammalian microsomes, too.

EXPERIMENTAL PROCEDURES

Materials—[³⁵S]Methionine (1000 Ci/mmol) was obtained from Amersham Buchler. BapEI was from New England BioLabs. All the other restriction enzymes, DNA modifying enzymes, RNase A, ATP, and the

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1 The abbreviations used are: PPIase, peptidylprolyl cis/trans-isomerase; RM, rough microsomes; PKRM, puromycin/high salt-treated rough microsomes; MsSO, dimethyl sulfoxide; ATP₄S, adenosine 5’-O-(thio) triphosphate; FKBP, FK506 binding proteins; CsA, cyclosporin A.
nonhydrolyzable ATP analog ATP-γ-S were purchased from Boehringer Mannheim and the coupled transcription/translation system were from Promega. Cycloheximide and potato apryase (grade VIII) were obtained from Sigma. X-ray films (X-Omat AR) were from Kodak.

Construction of Plasmids Coding for Luciferase-related Presecretory Proteins—DNA manipulations were carried out according to Sambrook et al. (1989). All plasmid constructs were checked by DNA sequencing according to the supplier's recommendations. Where indicated, dog pancreas microsomes or proteoliposomes were present during the translation. In order to prevent N-glycosylation of luciferases in microsomes, the acceptor peptide Ac-Asn-Tyr-Thr-NH$_2$ (final concentration: 0.1 mM) was present simultaneously.

Thus, in all constructs the original cleavage site of either preprocecropin A (Ala), the dipeptide Ac-Asn-Tyr-Thr-NH$_2$ (final concentration: 0.1 mM) was present simultaneously.

The various hybrid precursor proteins comprised (i) the preprocecropin A signal peptide, the first amino acid residue of procecropin A (Ala), the tetrapeptide Pro-Val-Asp-Val, and LuxA (coded for by plasmid pML19); (ii) the preprolactin signal peptide, the first amino acid residue of prolactin (Thr), the tetrapeptide Pro-Val-Asp-Val, and LuxA (coded for by plasmid pML20); (iii) the preprocecropin A signal peptide, the first amino acid residue of procecropin A (Ala), the dipeptide Pro-Asp, and LuxB (coded for by plasmid pML18). The various hybrid precursor proteins comprised (i) the preprocecropin A signal peptide, the first amino acid residue of procecropin A (Ala), the dipeptide Pro-Val-Asp-Val, and LuxA (coded for by plasmid pML20); (iii) the preprocecropin A signal peptide, the first amino acid residue of procecropin A (Ala), the dipeptide Pro-Asp, and LuxB (coded for by plasmid pML18). Therefore, all the original cleavage site of either preprocecropin A or proprolactin for signal peptide was preserved.

Reconstruction of Microsomal Membrane Proteins—Dog pancreas microsomes were prepared as described (Watts et al., 1983). The preparation of proteinoliposomes started from ribosome-depleted microsomes (PKRM), which were obtained by puromycin/high-salt treatment of rough microsomes (RM) according to the protocol of Görlich and Rapoport (1993). PKRM were adjusted to a concentration of 1 eq/μl in solubilization buffer (20 mM HEPES-KOH (pH 7.5), 400 mM KCl, 200 mM sucrose, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM dithiothreitol, and 17.5% (v/v) glycerol). Solubilization of membranes was achieved by addition of cholate (Calbiochem, 0.9% (w/v) final concentration) and gentle mixing. After 20 min on ice and centrifugation for 20 min in a TLA 100.3 rotor at 200,000 × g and 2 °C, the detergent extract was reconstituted into proteinoliposomes by incubation of 200-μl aliquots on a roller apparatus for 2–3 h at 4 °C with 100 mg of Bio-Beads SM2 (Bio-Rad) that had previously been washed with methanol (three times) and equilibrated with the solubilization buffer. After dilution with 400 μl of microsome dilution buffer (20 mM HEPES-KOH (pH 7.5), 50 mM KCl, 200 mM sucrose, 2 mM MgCl$_2$, 1 mM dithiothreitol) and separation of the fluid phase from the beads, the proteinoliposomes were collected by centrifugation for 30 min in a TLA 100.3 rotor at 200,000 × g and 2 °C. The proteinoliposomes finally were resuspended in 30 μl of microsome dilution buffer, i.e. at a concentration corresponding to 6.7 eq/μl.

In Vitro Transcription and Translation—The various plasmids were used to synthesize the respective proteins in a coupled transcription/translation system in the presence of [35S]methionine (final concentration, 1.4 μCi/ml) or in the presence of unlabeled methionine following the supplier's recommendations. Where indicated, dog pancreas microsomes or proteinoliposomes were present during the translation. In order to prevent N-glycosylation of luciferases in microsomes, the acceptor peptide Ac-Asn-Tyr-Thr-NH$_2$ (final concentration: 0.1 mM) was present simultaneously.

Thus, in all constructs the original cleavage site of either preprocecropin A (Ala), the dipeptide Ac-Asn-Tyr-Thr-NH$_2$ (final concentration: 0.1 mM) was present simultaneously.
peptide (data not shown).

In a first set of folding experiments the kinetics of synthesis of pLuxA and pLuxB were compared with the kinetics of luciferase folding (shown for synthesis of pLuxA and pLuxB in the presence of microsomes plus acceptor peptide in Fig. 1, B and C). In order to follow the efficiencies of synthesis and transport of the respective precursor polypeptide chains, the translation reaction was carried out in the presence of $[35S]$methionine. Aliquots of the translation/transport reactions were subjected to gel electrophoresis and fluorography (Fig. 1B). In order to follow the kinetics of luciferase assembly under these conditions, the translation was carried out in the absence of labeled amino acid. Aliquots were analyzed in the presence of the required substrates of the luciferase reaction by luminometry.

Fig. 1. Folding of LuxAB in dog pancreas microsomes. Plasmids pML18 and pML20 were used to synthesize pLuxA and pLuxB in a coupled transcription/translation system at 23 °C either in absence of rough microsomes (RM), or in the presence of microsomes, or in the presence of microsomes plus acceptor peptide (NYT). The concentration of microsomes corresponded to an absorbance at 280 nm of 6 (as measured in 2% sodium dodecyl sulfate). A, translation was carried out in the presence of $[35S]$methionine. After 25 min each translation reaction was divided into three aliquots. One aliquot was incubated further in the absence of protease, a second one in the presence of protease (final concentration of proteinase K, 200 μg/ml), and a third one in the presence of protease plus detergent (final concentration of Triton X-100, 0.5%) for 60 min at 0 °C. After inhibition of the protease by addition of phenylmethylsulfonyl fluoride (final concentration, 10 mM) and incubation for 5 min at 0 °C, all samples were analyzed by gel electrophoresis and fluorography (pLux, precursor; Lux, mature protein; gLux, glycosylated protein). B, translation was carried out in the presence of $[35S]$methionine. At the indicated times aliquots were withdrawn and analyzed for protein content (filled squares, pLuxA plus LuxA; open squares, pLuxB plus LuxB) by gel electrophoresis, fluorography, and densitometry (arbitrary units). The data from the translation in the presence of microsomes and acceptor peptide are shown. C, translation was carried out in the presence of unlabeled methionine. At the indicated times aliquots were withdrawn and analyzed for luciferase activity, given as relative light units $\times 10^{-2}$. The data from the translation in the presence of microsomes plus acceptor peptide are shown (filled circles). D, translation was carried out in the presence of unlabeled methionine. After 300 min each folding reaction was divided into three aliquots. One aliquot was incubated further in the absence of protease (1), a second one in the presence of protease (final concentration of trypsin, 30 μg/ml) (2), and a third one in the presence of protease plus detergent (3). After inhibition of the protease by addition of leupeptin (final concentration, 420 μM) plus phenylmethylsulfonyl fluoride (final concentration, 10 mM) and incubation for 5 min at 0 °C, all samples were analyzed for luciferase activity, given as relative light units $\times 10^{-2}$. The data from the translation in the presence of microsomes plus acceptor peptide are shown (filled circles). E, translation was carried out in the presence of unlabeled methionine. After 25 min (indicated by arrow) cycloheximide (final concentration, 100 μg/ml) and RNase A (final concentration, 80 μg/ml) were added to stop the translation reaction, and the folding reaction was allowed to proceed. At the indicated times aliquots were withdrawn and analyzed for luciferase activity, given as relative light units $\times 10^{-2}$ (filled circles, no addition; open diamonds, RM; filled triangles, RM + NYT). F, translation was carried out in the presence of $[35S]$methionine. At the indicated times aliquots were withdrawn and divided into two aliquots. One aliquot was incubated further in the absence of protease (data not shown) and the second one in the presence of proteinase K (final concentration, 25 μg/ml) plus detergent. All samples were analyzed by gel electrophoresis, fluorography, and densitometry (arbitrary units). The data from the translation in the presence of microsomes and acceptor peptide are shown (open squares, protease-resistant LuxB; filled squares, protease-resistant LuxA).
We observed that the rate of LuxAB-assembly (half-time, about 70 min) was low when compared with the rates of protein synthesis and transport (half-time, about 30 min) (Fig. 1, B versus C). In order to determine the location of the enzymatically active luciferase, the translation was carried out in the absence of a labeled amino acid, and aliquots were subjected to sequestration analysis and subsequent luminometry (shown for synthesis of pLuxA and pLuxB in the presence of microsomes plus acceptor peptide in Fig. 1D). We observed that the enzymatically active luciferase was completely sequestered, i.e. the enzyme activity was resistant to protease in the absence of detergent and protease-sensitive in the presence of detergent (Fig. 1D).

In a subsequent experiment 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 (Kruse et al., 1995). The precursors pLuxA and pLuxB were synthesized in the absence of microsomes, in the presence of microsomes, or in the presence of microsomes plus acceptor peptide. After 25 min the translation/transport reaction was inhibited, and the incubation for folding was continued. Aliquots were analyzed by luminometry. Assembly of luciferase occurred only in the presence of microsomes plus acceptor peptide (Fig. 1E). Apparently, the presence of the signal peptides as well as the glycosylation of the mature proteins prevented the folding and/or assembly reaction (see Fig. 1D).

In the course of the transport experiments in the presence of acceptor peptide, it became apparent that at proteinase K concentrations (25 μg/ml) that were lower as compared with the sequestration analysis (200 μg/ml), LuxA and LuxB were protease-resistant even in the presence of detergent to a certain degree (data not shown). Therefore, we asked whether protease-resistant LuxA and/or LuxB that was observed under these conditions (i.e. in the presence of detergent) reflects native LuxAB. The precursors pLuxA, pLuxB, or pLuxA plus pLuxB were synthesized in the presence of [35S]methionine, either in the absence or presence of microsomes and acceptor peptide. Aliquots of the translation/transport reactions were taken and subjected to protease treatment in the presence of detergent, followed by gel electrophoresis and fluorography. After separate or simultaneous synthesis of pLuxA and pLuxB in the absence of microsomes, protease-resistant polypeptides were not detected. While after separate synthesis of pLuxA and pLuxB in the presence of microsomes and acceptor peptide, only LuxB gained protease resistance to a certain degree (data not shown), both subunits showed a significant level of protease resistance after simultaneous synthesis of pLuxA and pLuxB in the presence of microsomes and acceptor peptide (Fig. 1F).

The kinetics of the formation of the protease-resistant form of LuxAB (half-time, about 65 min) coincided with the kinetics of the formation of enzymatically active enzyme (half-time, about 70 min) (Fig. 1, F versus C). Furthermore, enzymatically active enzyme that had been formed after simultaneous synthesis of pLuxA and pLuxB in the presence of microsomes and acceptor peptide was completely resistant against low levels of proteinase K in the presence of detergent (data not shown). Therefore, we propose that protease resistance of LuxA in the presence of detergent at low protease concentrations can be used as an additional demonstration for folding of LuxA to the native state, i.e. assembly of LuxAB. Taking into account that the luciferase activity was completely protease-resistant under these conditions (see above), one can estimate from these data that about 40% of the LuxA polypeptides reached the native state in the microsomal lumen.

Thus folding of the bacterial luciferase to the native state occurred after synthesis of the two subunits in reticulocyte lysate and concomitant transport into dog pancreas microsomes. Furthermore, the assembly of luciferase could be separated in time from the synthesis and transport of the two polypeptides. Similar results were obtained with precursor proteins that contained the preprocecropin A signal peptide instead of the preprolactin signal peptide (data not shown). Therefore, the bacterial luciferase provides a suitable system for studying the proteolytic folding and assembly in mammalian microsomes.

Folding of Bacterial Luciferase in Mammalian Microsomes Involves ATP Hydrolysis—In order to investigate the potential involvement of molecular chaperones in luciferase assembly in dog pancreas microsomes, the folding kinetics of luciferase were studied after supplementing the folding mixture with inhibitors of ATP-dependent molecular chaperones. The folding reactions were analyzed with respect to their sensitivities to ATP depletion. Furthermore, we asked whether the addition of ATP or a nonhydrolyzable analog of ATP (i.e. ATP·S) suppress the effect of ATP depletion and whether ATP·S interferes with luciferase assembly. We note that ATP·S was previously shown to be a substrate for the ATP transporter in the microsomal membrane (Clairmont et al., 1992).

The precursors pLuxA and pLuxB were synthesized for 23 min in the presence of microsomes plus acceptor peptide, and then protein synthesis and concomitant transport were inhibited, and the transport mixtures were divided into several aliquots. A first aliquot was left untreated, and a second aliquot was supplemented with apyrase, an enzyme that catalyzes ATP and ADP hydrolysis (Fig. 2A). In addition, a third aliquot was supplemented with apyrase plus ATP, and a fourth aliquot was supplemented with apyrase plus ATP·S (Fig. 2B). Furthermore, a fifth aliquot was supplemented with ATP (Fig. 2B), and a sixth aliquot was supplemented with ATP·S (Fig. 2A). The incubation was continued, and the enzyme activities were monitored. Assembly of LuxAB (Fig. 2B) was sensitive to apyrase treatment. The inhibitory effect of apyrase depended on the apyrase concentration (data not shown) and reached a level of 25% inhibition under these conditions. The effect of apyrase was due to depletion of ATP since the apyrase effect was prevented by ATP but not by ATP·S (Fig. 2B). The inhibitory effect of apyrase plus ATP·S reached a level of 40% inhibition. Furthermore, it was found that ATP·S competed with ATP and had a similar inhibitory effect on assembly of LuxAB as compared with apyrase (Fig. 2A). When apyrase was added after completion of LuxAB assembly, it had no effect on the enzyme activity (data not shown). Therefore, these experiments strongly suggest that ATP depletion did not interfere with the catalytic activity of luciferase but affected its folding. Sequestration analysis, carried out after 180 min of folding and prior to the luciferase assay, confirmed that effects of ATP depletion and ATP·S on luciferase folding in the microsomes were analyzed under these conditions (data not shown). Furthermore, the effects of ATP depletion and of ATP·S were also observed when protease sensitivity of LuxA was analyzed in the presence of detergent (data not shown).

On the basis of these results we conclude that assembly of heterodimeric luciferase in mammalian microsomes involves the hydrolysis of ATP. During our previous analysis of luciferase assembly after synthesis of LuxA and LuxB in rabbit reticulocyte lysate, we reached the conclusion that the ATP-dependent step occurs early in the folding of the two subunits, i.e. prior to the association of the two subunits (Kruse et al., 1995). Therefore, we suggest that this is also true for luciferase as-

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2 J. Solsbacher and R. Zimmermann, unpublished results.
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Folding of Bacterial Luciferase in Mammalian Microsomes Involves Peptidyl-Prolyl cis/trans-Isomerases—The PPIases include two structurally distinct protein families, the cyclophilins and high affinity binding sites for the drug cyclosporin A (CsA), whereas the FK506 binding proteins (FKBPs) bind FK506 (Schreiber, 1991; Kunz and Hall, 1993). In both cases, the FK506 binding proteins (FKBPs) bind FK506 (open squares), the fifth one with apyrase plus ATP (B, open diamonds), and the sixth one with apyrase plus ATPγS (B, filled circles). Subsequently, the folding reaction was allowed to proceed. At the indicated times aliquots were withdrawn and analyzed for luciferase activity, given as relative light units $\times 10^{-3}$.

assembly in microsomes and accounts for the moderate effects of ATP depletion and ATPγS.

Folding of Bacterial Luciferase in Mammalian Microsomes Involves Peptidyl-Prolyl cis/trans-Isomerases—The PPIases include two structurally distinct protein families, the cyclophilins and high affinity binding sites for the drug cyclosporin A (CsA), whereas the FK506 binding proteins (FKBPs) bind FK506 (Schreiber, 1991; Kunz and Hall, 1993). In both cases, binding of the drug inhibits isomerase activity of the enzyme.

The assembly kinetics of luciferase were studied after pretreatment of microsomes with CsA and FK506. This pretreatment did not interfere with subsequent protein synthesis and transport (data not shown). The precursors pLuxA and pLuxB were synthesized in the presence of microsomes plus acceptor peptide and in the additional presence of Me$_2$SO, CsA, or FK506 (Fig. 3). The enzyme activities were measured. Folding of LuxAB was partially sensitive to each of the two drugs. When addition of CsA or FK506 occurred after completion of folding of LuxAB 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 luciferase but rather affected its assembly. We concluded from these data that PPIases may be involved in assembly of LuxAB. We therefore expected that simultaneous inhibition of both types of PPIases results in a more pronounced inhibition of luciferase assembly. Thus, the assembly kinetics were studied after microsomes had been pretreated with the two inhibitors at the same time. Assembly of LuxAB was found to be more sensitive to a combined addition of CsA and FK506 as compared with the addition of CsA or FK506 alone, therefore corroborating a possible role of PPIases in the assembly of newly synthesized and transported luciferase subunits. Sequestration analysis, carried out after 300 min of folding and prior to the luciferase assay, confirmed that effects of CsA and FK506 on luciferase folding in the microsomes were analyzed under these conditions (data not shown). Furthermore, the effects of CsA and of FK506 were also observed when protease sensitivity of LuxA was analyzed in the presence of detergent (data not shown). We conclude that PPIases may be involved in assembly of LuxAB in mammalian microsomes.

Folding of Bacterial Luciferase in Proteoliposomes—To corroborate the involvement of ATP-dependent molecular chaperones and PPIases in luciferase assembly in the microsomes, the folding kinetics of luciferase were analyzed after transport of the two subunits into proteoliposomes, i.e., in the absence of luminal molecular chaperones and folding catalysts. First transport of the luciferases into proteoliposomes was studied. In a second set of experiments, we asked whether the absence of luminal proteins influences luciferase assembly. In a third and fourth set of experiments, we asked whether ATP depletion or addition of PPIase inhibitors interferes with the assembly reactions in proteoliposomes.

In the first set of experiments, the precursors pLuxA and pLuxB were synthesized in the presence of proteoliposomes plus acceptor peptide. In order to follow the efficiencies of transport of the respective precursor polypeptide chains, the translation reaction was carried out in the presence of [35S]methionine. Aliquots of the translation reactions were subjected to sequestration analysis, gel electrophoresis, and fluorography (data not shown). After synthesis of pLuxA and pLuxB in the presence of proteoliposomes, the precursor proteins and two additional polypeptides were detected that had a lower apparent molecular mass as compared with the precursor proteins, pLuxA and pLuxB, and had the apparent molecular mass of the corresponding native proteins, LuxA and LuxB. Thus, in the presence of proteoliposomes the precursor proteins were processed by signal peptidase. Additionally, these data can be taken as an indication for the fact that transport of the precursor proteins into the proteoliposomes had occurred. This inter-
pretation was confirmed by the observations that the mature proteins were resistant to protease in the absence of detergent and protease-sensitive in the presence of detergent.

In the second set of experiments the precursors of LuxA and LuxB were synthesized in the presence of acceptor peptide, either in the presence of microsomes or in the presence of proteoliposomes. In order to follow the efficiencies of transport of the respective precursor polypeptide chains, the translation reaction was carried out in the presence of $[^{35}S]$methionine. Aliquots of the translation reactions were subjected to sequestration analysis, gel electrophoresis, and fluorography (Table I). We note that for these experiments the concentration of microsomes was lowered as compared with the previous experiments in order to compare similar transport efficiencies for microsomes and proteoliposomes. To follow the kinetics of luciferase assembly, the translation was carried out in the absence of a labeled amino acid, and aliquots were subjected to sequestration analysis, gel electrophoresis, and fluorography (Fig. 4). The enzyme activities were measured. Folding of LuxAB in proteoliposomes was insensitive to the two drugs. One aliquot was supplemented with water (filled squares) and the second one with apyrase (20 units/ml) (open squares). Subsequently, the folding reaction was allowed to proceed. At the indicated times aliquots were withdrawn and analyzed for luciferase activity, given as relative light units $\times 10^{-3}$. Folding of LuxAB in proteoliposomes was insensitive to the two drugs.

Therefore, we conclude that at least a significant proportion of the inhibitory effects of ATP depletion, CsA and FK506 on luciferase assembly in dog pancreas microsomes is due to an inhibition of ATP-dependent luminal proteins and luminal PPLases and not due to either direct or indirect effects of the various agents on the luciferase polypeptides.

### DISCUSSION

Little is known about the general rules and mechanisms governing protein folding and subunit assembly in the various compartments of the eukaryotic cell, following de novo synthesis of polypeptides (Ellis and van der Vies, 1981; Gething and Sambrook, 1992; Georgopoulos and Welch, 1993; Hartl et al., 1994). However, folding and assembly of complex proteins, i.e., proteins that contain disulfide bridges and/or sugar moieties, within the endoplasmic reticulum is reasonably well understood (Gething et al., 1994; Bergeron et al., 1994; Helenius et al., 1994). Here, we describe an in vitro system that allows general studies on protein folding and assembly in mammalian microsomes by using an enzyme that should be free of sugar moieties and disulfide bridges in its natural habitat. The het-
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erodimeric luciferase was chosen as a model enzyme because its enzymatic activity is easily detected and because of the available information about its requirements for folding and assembly after synthesis of the two subunits in E. coli cells (Waddle et al., 1987; Olsson et al., 1988; Escher et al., 1989; Flynn et al., 1993; Ziegler et al., 1993; Escher and Szalay, 1993) and rabbit reticulocyte lysate (Kruse et al., 1995), respectively.

Here, we demonstrate that efficient assembly of this luciferase occurs after synthesis of the two subunits in a cell-free translation system and their concomitant transport into dog pancreas microsomes. The following was observed. (i) Luciferase assembly can be separated in time from synthesis and transport. (ii) Assembly depends on the hydrolysis of ATP. (iii) Assembly involves lumenal proteins that depend on ATP for their action. Therefore, we conclude that assembly of luciferase in mammalian microsomes involves ATP-dependent molecular chaperones. The most likely explanation for the observed ATP effect is that immunoglobulin heavy chain-binding protein may be involved in the folding of this luciferase.

Peptidyl-prolyl cis-trans-isomerases (PPIases) catalyze conversion between cis- and trans-isomers of proline-containing peptide bonds in vitro and, typically, accelerate these slow folding steps. PPIases are abundant proteins and belong to either one of two related protein families that can be distinguished by their sensitivity to the drugs CsA and FK506 (Lang et al., 1989; Fischer et al., 1989). Previous observations pointed to a role of cyclophilins in protein folding in the endoplasmic reticulum. CsA inhibits the folding of type I collagen (Davis et al., 1989; Steinmann et al., 1991) and transferin (Lodish and Kong, 1991) in the endoplasmic reticulum of fibroblasts and Hep G2 cells, respectively. In Drosophila melanogaster the cyclophilin homolog ninaA is essential for transport of rhodopsin through the secretory pathway (Colley et al., 1991). Here, we asked if the assembly of heterodimeric luciferase after de novo synthesis of the two subunits in rabbit reticulocyte lysate and their concomitant transport into dog pancreas microsomes involves endogenous PPIases. The following observations were made. (i) Luciferase assembly is partially sensitive to CsA and FK506. (ii) A combination of CsA and FK506 leads to a more pronounced inhibition of assembly. (iii) The inhibitory effect of CsA and FK506 on assembly cannot be observed in proteoliposomes, i.e. in the absence of PPIases. Therefore, we conclude that assembly of luciferase in mammalian microsomes involves lumenal PPIases, cyclophilins, as well as FKBP’s that are present in the microsomes (Price et al., 1991; Kunz and Hall, 1993; Bose and Freedman, 1994; Bose et al., 1994). Thus, heterodimeric luciferase provides the first direct evidence documenting the involvement of FKBP’s in protein biogenesis in the endoplasmic reticulum. It is unclear why inhibition of PPIases led to an inhibition of luciferase assembly in both the cytosol (Kruset al., 1995) and the microsomal lumen (shown here), whereas PPIases have been observed to increase the rate of folding. It seems possible that upon inhibition of PPIases one or both subunit(s) of heterodimeric luciferase is/are prone to aggregation.

An additional important conclusion can be reached from comparing the studies that were reported here and our previous studies on assembly of the same model protein in rabbit reticulocyte lysate (Kruse et al., 1995). The requirements of a certain protein for folding and assembly are determined by the protein and not by the available chaperones and folding catalysts, i.e. the intracellular compartment.

Implications for Protein Transport into and for Protein Folding in the Endoplasmic Reticulum—The apparent dependence of luciferase assembly on molecular chaperones and folding catalysts should allow us in future studies to include defined members of these two protein families in the proteoliposomes and, thereby, allow us to identify the relevant proteins and to understand their role in luciferase assembly. The bacterial molecular chaperone GroEL was previously shown to be able to preserve the assembly-competent state of the two subunits of the heterodimeric enzyme after separate synthesis in Escherichia coli cells (Escher and Szalay, 1993; Flynn et al., 1993). Under the assumption that there is no Hsp60 homolog in the microsomal lumen, it should be interesting to see which of the microsomal chaperones can functionally substitute for Hsp60 in the microsomes. The bacterial luciferase is a non-glycoprotein and, as cytosolic protein, can be expected to be free of disulfide bridges. Thus it allows us to study protein folding in the endoplasmic reticulum independently of these covalent modifications. Therefore, the potential general chaperoning activity of molecular chaperones that have been shown to be involved in folding of glycoproteins, such as calnexin (Burgeren et al., 1994), should be accessible to future analysis. Furthermore, the putative chaperoning activity of protein disulfide isomerase may be studied under these conditions (LaMantia and Lennarz, 1993).

The cotranslational mode of transport of proteins into the mammalian endoplasmic reticulum is reasonably well understood by now. However, it was shown that the ATP-dependent molecular chaperone immunoglobin heavy chain-binding protein is directly involved in completion of translocation in lower eukaryotes (Sanders et al., 1992) and that the hydrolysis of ATP (Klappa et al., 1991) and lumenal proteins (Niechitta and Blobel, 1993) are required for translocation in higher eukaryotes. However, the nature of the ATP-dependent and lumenal protein(s) and its/their exact role in translocation are still obscure. By having observed a positive effect of one or more ATP-dependent lumenal protein(s) on protein folding, we should eventually be able to answer the question of whether this/these protein(s) also plays a role in protein transport.

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