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Folding of firefly luciferase during translation in a cell-free system

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***In vitro* synthesis of firefly luciferase and its folding into an enzymatically active conformation were studied in a wheat germ cell-free translation system. A novel method is described by which the enzymatic activity of newly synthesized luciferase can be monitored continuously in the cell-free system while this protein is being translated from its mRNA. It is shown that ribosome-bound polypeptide chains have no detectable enzymatic activity, but that this activity appears within a few seconds after luciferase has been released from the ribosome. In contrast, the renaturation of denatured luciferase under identical conditions occurs with a half-time of 14 min. These results support the cotranslational folding hypothesis which states that the nascent peptides start to attain their native tertiary structure during protein synthesis on the ribosome.**

Key words: luciferase/nascent peptide/protein folding/ribosome

Introduction

The problem of protein folding has been the subject of considerable discussion for over four decades. A number of experimental approaches have been developed to investigate how proteins attain their unique tertiary structure. The great majority of these studies are based on the examination of renaturation of mature proteins from the denatured state (Anfinsen, 1973; Wetlaufer, 1981). This methodology has provided a basic insight into the principles of protein structure arrangement (Anfinsen and Scheraga, 1975; Jaenicke, 1988; Kuwajima, 1989; Kim and Baldwin, 1990). Rapid and complete renaturation from the fully unfolded state was demonstrated for several small proteins including RNase A (Anfinsen and Haber, 1961), staphylococcal nuclease (Epstein *et al.*, 1971), cytochrome *c* (Ikai and Tanford, 1971) and lysozyme (Tanford *et al.*, 1973). Considering such experimental data, Anfinsen and Scheraga (1975) and, more recently, Creighton (1984) reached the conclusion that protein renaturation *in vitro* is similar to folding *in vivo*.

However, there are several theoretical reasons which cast some doubt on this point of view. (i) Synthesis of polypeptide chain progresses vectorially from its N- to its C-terminus, suggesting that the N-terminal part could begin to fold during the course of translation. This assumption appears to be quite realistic, since oligopeptides as short as 10–20 amino acid residues have been shown to attain native secondary structure in solution (Wright *et al.*, 1988). Furthermore, polypeptides corresponding to protein domains are capable of forming the correct tertiary structure that occurs in the native protein (Teale and Benjamin, 1977). (ii) The attachment of the nascent protein's C-terminus to the large mass of the ribosome during translation may affect the pathway and the rate of three-dimensional structure formation (Spirin, 1986). (iii) The ribosome must generate polypeptide chains with a universal initial conformation of amino acid residues which can only be α-helical (Lim and Spirin, 1986). If this is the case, protein folding *in vivo* does not begin from the 'random coil' state, which is what takes place during renaturation *in vitro*.

In addition, there is a body of experimental evidence contradictory to the assumption that protein folding is completely post-translational. For instance, many proteins take tens of minutes or even hours to become renatured. Such long periods are unrealistic for the living cell, which contains many proteins with much shorter half-lives (Dice and Goldberg, 1975). Summarizing arguments such as those mentioned above it has been presumed that proteins *in vivo* start folding during translation while they are still attached to the ribosome (Chantrenne, 1961; Phillips, 1967; DeCoen, 1970; Freedman, 1992; Gething and Sambrook, 1992). Unfortunately, there are practically no direct data supporting this hypothesis because of the difficulties encountered in this type of experimental work.

To approach this problem we developed a novel technique for continuously monitoring the enzymatic activity of a newly synthesized protein in a cell-free system. Our studies were based on the accepted assumption that only a correctly folded protein possesses biological activity whereas an unfolded, incompletely folded or misfolded protein does not. The enzyme we chose was luciferase from the firefly *Photinus pyralis*. Luciferase catalyzes the oxidation of a specific heterocyclic compound, luciferin, with molecular oxygen in the presence of ATP and Mg^{2+}. This reaction results in light emission with a peak at 560 nm (DeLuca and McElroy, 1978).

Results

Translation of luciferase mRNA in the wheat germ cell-free system results in the synthesis of active luciferase

Luciferase mRNA (lucRNA) was transcribed from a plasmid carrying the coding region of luciferase cDNA under the control of the SP6 promoter. This mRNA was translated *in vitro* using the wheat germ extract system (Hames and Higgins, 1984). After incubating the translation mixture at 25°C for 75 min, aliquots were removed

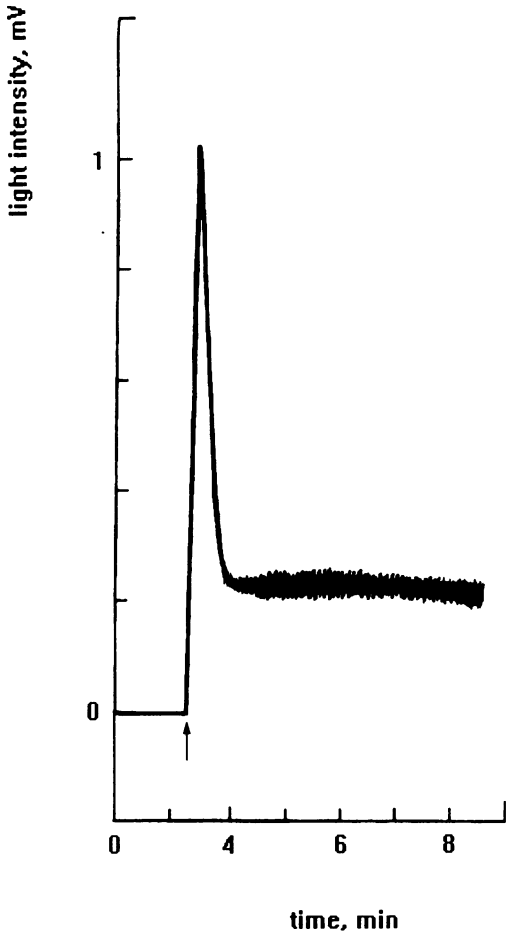

Fig. 1. Luciferase activity assay of the wheat germ translation mixture. The activity was measured by emission of light with time upon luciferin addition. Five microliters of the translation mixture containing lucRNA was removed after 75 min incubation at 25°C and assayed according to the standard procedure (see Materials and methods). The arrow indicates the time of luciferin addition. Translation mixture without lucRNA showed no activity in this assay (not shown).

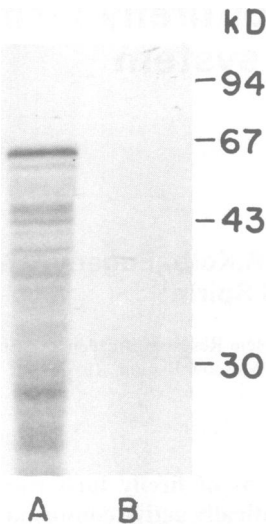

Fig. 2. SDS–PAGE of *in vitro* translation products. Aliquots of 5 µl were removed from translation mixtures after 75 min incubation at 25°C and subjected to electrophoresis in a 10% polyacrylamide gel (Laemmli, 1970) followed by autoradiography. Lane A is from reaction mixture with luciferase mRNA; lane B is the control without mRNA.

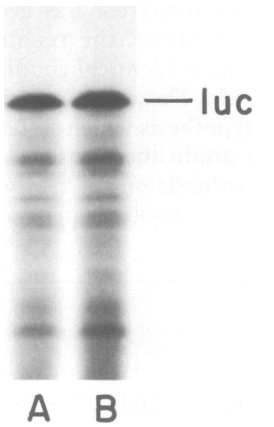

Fig. 3. Autoradiogram of the gel containing *in vitro* translation products. Translation and electrophoresis conditions were as described in the legend to Figure 2. Lane A is from the reaction mixture with lucRNA and without luciferin; lane B is from the mixture where lucRNA was translated in the presence of 0.1 mM luciferin.

and assayed for luciferase activity according to a commonly used procedure in a luminometer equipped with a chart recorder (DeLuca and McElroy, 1978). As seen from the typical course of light emission upon luciferin addition (Figure 1), luciferase activity was detected in the case of lucRNA translation whereas no activity was observed in the control without mRNA (not shown). Aliquots of the same translation mixtures—with and without lucRNA—were subjected to SDS–PAGE in 10% polyacrylamide gels and the products were visualized by autoradiography (Figure 2). The major product of lucRNA translation was a protein migrating like firefly luciferase (~62 kDa). The control did not contain any significant bands.

Accumulation of the full-sized luciferase correlates temporally with the increase in light production

In subsequent investigations we used an unconventional luciferase assay to establish exactly when enzymatic activity appeared in the course of translation. In these experiments, up to 0.1 mM luciferin was added directly to the translation mixture before incubation at 25°C. At such a concentration luciferin affects neither the efficiency of lucRNA translation nor the size distribution of the translational products, as evident from the corresponding electrophoretic pattern (Figure 3). The other substrates of luciferase—ATP and Mg^{2+}—were present in the mixture as components of the cell-free system at concentrations of 1 and 2 mM, respectively. The assay was performed in the following order. After addition of luciferin an aliquot of the translation mixture was placed into a thermostated luminometer cell at 25°C. Light emission was recorded throughout the experiment (usually for 75 min). The rest of the translation mixture was also kept at 25°C in a separate test tube. Aliquots from this tube were removed at 3 min intervals and then subjected to SDS–PAGE in a 15% polyacrylamide gel. As is evident from the kinetics of the active luciferase accumulation (Figure 4A), there was no enzymatic activity during the first 18 min of

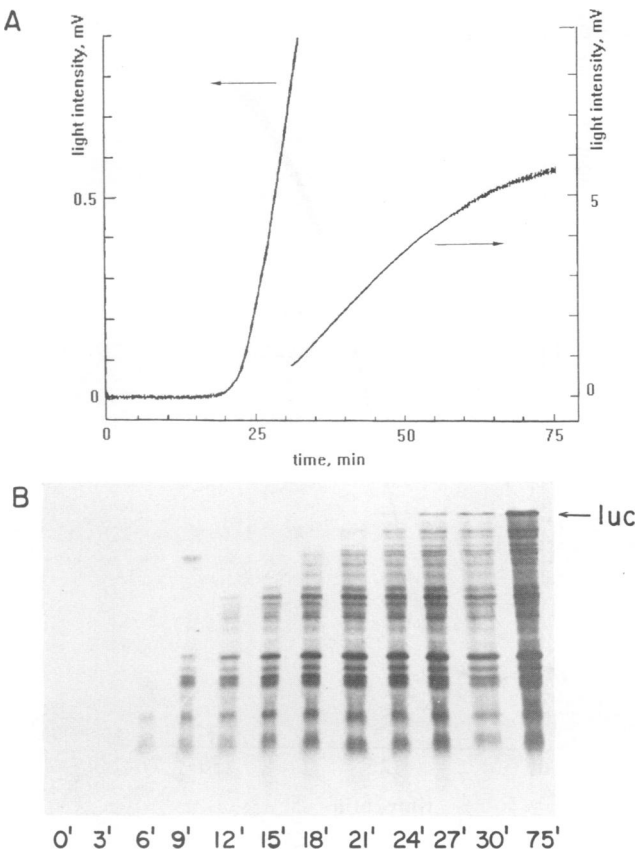

Fig. 4. (**A**) Time course of the accumulation of active luciferase during translation. Translation of the lucRNA was performed in a luminometer cell in the presence of 0.1 mM luciferin. The volume of translation mixture was 25 µl. The light emission during the course of translation was recorded. Detectable activity of the enzyme appeared after 18 min of incubation at 25°C. After 30 min the luminometer was switched from the 1 mV scale to the 10 mV scale. (**B**) Autoradiogram of gel electrophoresis of 5 µl aliquots sampled from the luciferin-containing translation mixture every 3 min from 0 to 30 min and then after 75 min. Electrophoresis was carried out in a 15% polyacrylamide gel according to Laemmli (1970). Mature luciferase (indicated by arrow) was not detected until 21 min.

incubation. Detectable activity appeared after this period and in a time-dependent manner. Luciferase activity thus correlated with the appearance of a signal corresponding to mature luciferase on the autoradiogram (Figure 4B). Moreover, the increase in light emission was accompanied by an increase in the width of the corresponding band on the autoradiogram. This result indicates that the obtained light emission curve reflects accumulation of mature active luciferase during translation. It also suggests that a very short period of time is needed for the newly synthesized luciferase to attain the active conformation.

Luciferase mRNA translation arrest leads to the immediate cessation of luciferase activity increase

To test the last assumption further, lucRNA was translated in a luminometer in the presence of luciferin as described above. When the synthesized luciferase activity was sufficiently high, RNase A solution in buffer A was added to the translation mixture to give a concentration of 1 mg/ml. This treatment abruptly stopped protein synthesis in the system. The curves of the active luciferase accumulation in this sample and a control treated with buffer A only are shown in Figure 5. The points at which RNase A or buffer solution were added are indicated by arrows. Accumulation of the active luciferase stopped immediately (within a few seconds) after translation had been arrested by RNase A. In contrast, no change was seen after addition of buffer alone. Thus, luciferase polypeptide chains just released from the ribosome have a fully active conformation or are completely folded within several seconds.

Renaturation of denatured luciferase is a slow process

The next experiment was conducted to determine whether the luciferase molecule folds predominantly on the ribosome during translation or whether it is rapidly folded after release from the ribosome. Luciferase, isolated from *Photinus pyralis* according to the procedure described previously (Green and McElroy, 1956), was denatured by incubating it for 10 min at 25°C in buffered 8 M urea or in ammonia solution, pH 11. Aliquots of the denatured enzyme solutions were diluted 20- to 500-fold into a wheat germ cell-free system containing 0.1 mM of luciferin and no mRNA. The test tube containing the wheat germ system was vortexed vigorously during the injection of denatured luciferase. Luciferase renaturation, which occurred under the above conditions, was carried out in a thermostated luminometer cell at 25°C. The time-dependent renaturation was measured by the recovery of light emission activity (Figure 6A and B). Similarly, aliquots of the non-denatured active luciferase were diluted with translation mixture as a control (Figure 6C). The average half-time of renaturation was 14 min both in the case of denaturation by urea (A) and by ammonia (B). The rate of renaturation was independent of the luciferase concentration and the dilution. The concentration of the renatured enzyme affected only the activity recovered: from ~20% at 50 µg/ml to 60% at 4 µg/ml (data not shown).

We have also carried out experiments to evaluate the effect of X-Pro peptide bond *cis–trans* isomerization on the rate of refolding of the denatured enzyme. We used the double jump procedure, in which the protein is rapidly transformed into the unfolded state and then returned to native conditions through variable delay times (McPhie, 1982; Semisotnov *et al.*, 1990). In more detail, renaturation of luciferase was initiated over 8–600 s delay times after fast unfolding in 8 M urea. In different experiments 2 mg/ml luciferase solution was diluted 20-fold into buffered 8 M urea at 4 or 25°C. Aliquots from the obtained mixture were removed following the delay times indicated above and diluted 30-fold with luciferin-containing wheat germ mixture. The rates of enzyme reactivation remained the same regardless of the temperature and duration of incubation with denaturant (data not shown). This result indicates that the *cis–trans* isomerization of X-Pro peptide bonds is not the rate-limiting step during luciferase refolding.

It is readily seen that refolding of the fully denatured luciferase proceeds more slowly than the few seconds needed for gaining activity after release of the enzyme from the ribosome. This phenomenon suggests that luciferase begins to take up its natural tertiary structure while it is still on the translating ribosome, and probably almost completes its folding there.

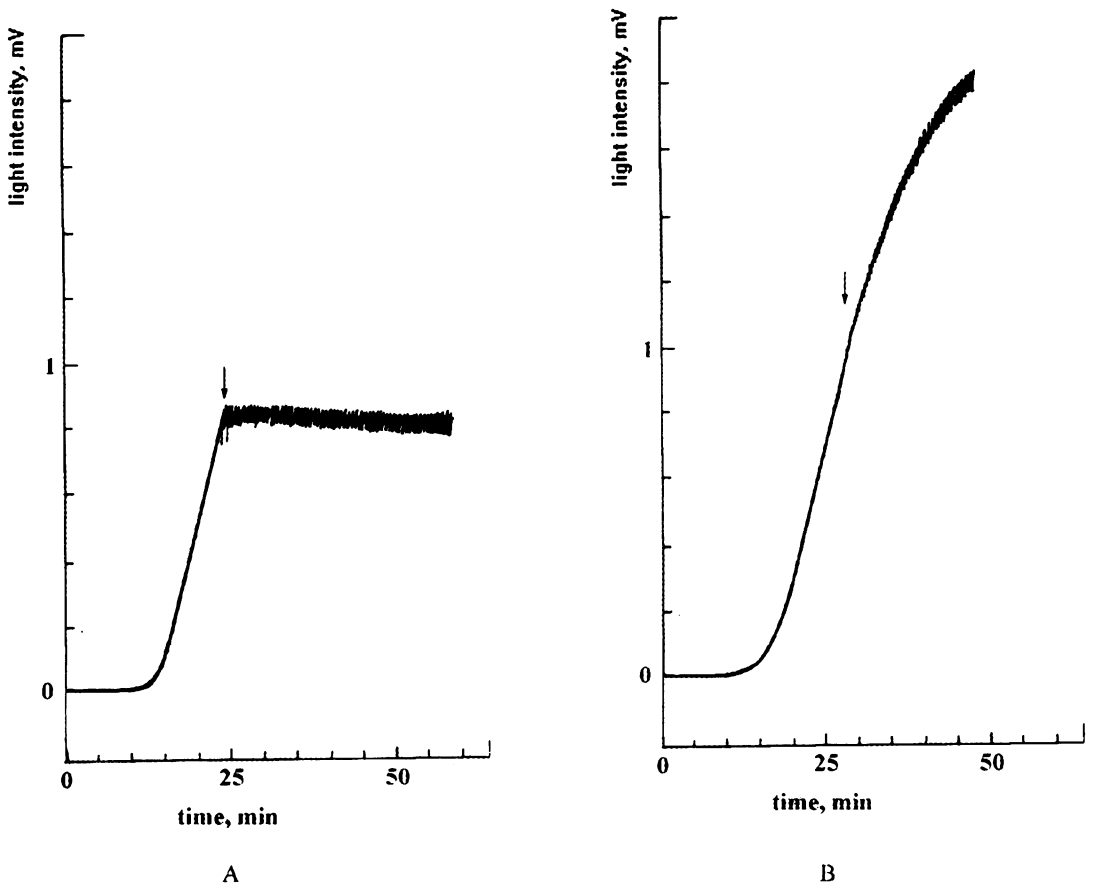

Fig. 5. Effect of RNase A on the translation of lucRNA. Luciferase mRNA was translated in the presence of luciferin as described in the legend to Figure 4. The volume of the translation mixture was 25 μl. At the moment indicated by the arrow (**A**) 2.5 μl of 10 mg/ml RNase A buffered solution or (**B**) 2.5 μl of the buffer A were injected into the translation mixtures. Active luciferase stopped accumulating immediately after RNase A addition.

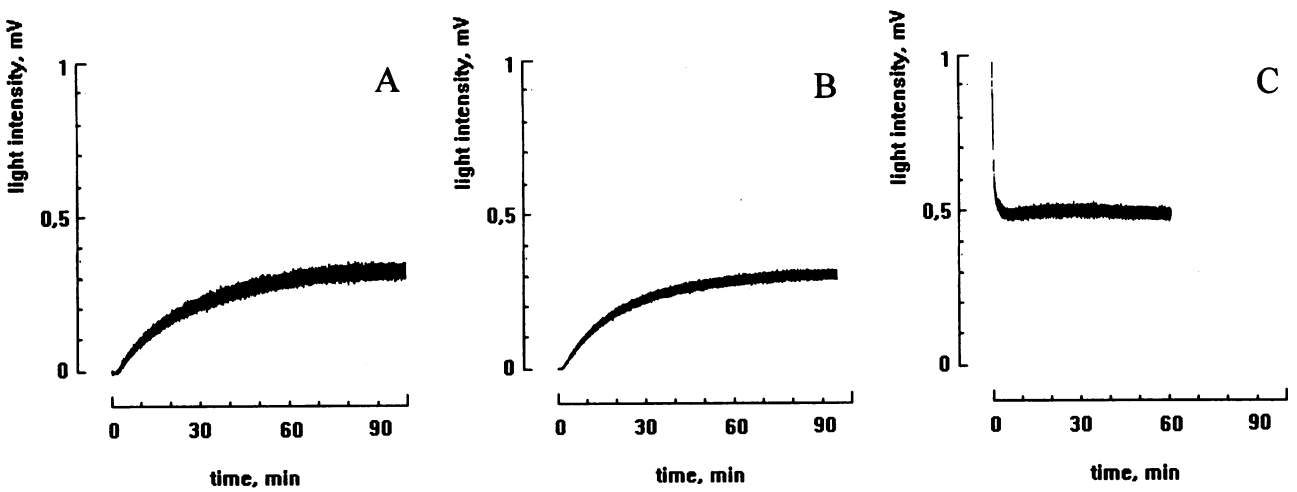

Fig. 6. Renaturation curves of (**A**) urea- and (**B**) ammonia-denatured luciferase. Luciferase solutions (2 mg/ml) in 8 M urea or in ammonia (pH 11) were diluted 500-fold into a wheat germ translation mixture containing 0.1 mM luciferin and no mRNA. Time course of the recovered activity was recorded. (**C**) Luciferase solution (2 mg/ml) in buffer A was diluted into the translation mixture as a control.

Ribosome-bound luciferase becomes active as soon as it is released

We have also tested whether luciferase can be active while still attached to the ribosome. For this purpose, truncated lucRNA was translated in a luminometer cell as described above. Luciferase mRNA depleted of one 3′-terminal coding triplet was obtained by treating lucRNA with RNase H in the presence of proper antisense 20 nt oligomer. Thus, the resulting mRNA had no stop codon in the luciferase open reading frame. Translation of mRNAs, truncated by RNase H in the presence of oligonucleotides complementary to the coding region results in nascent polypeptide attached to the ribosome, as was reported previously (Haeuptle *et al.*, 1986). Luciferase mRNA

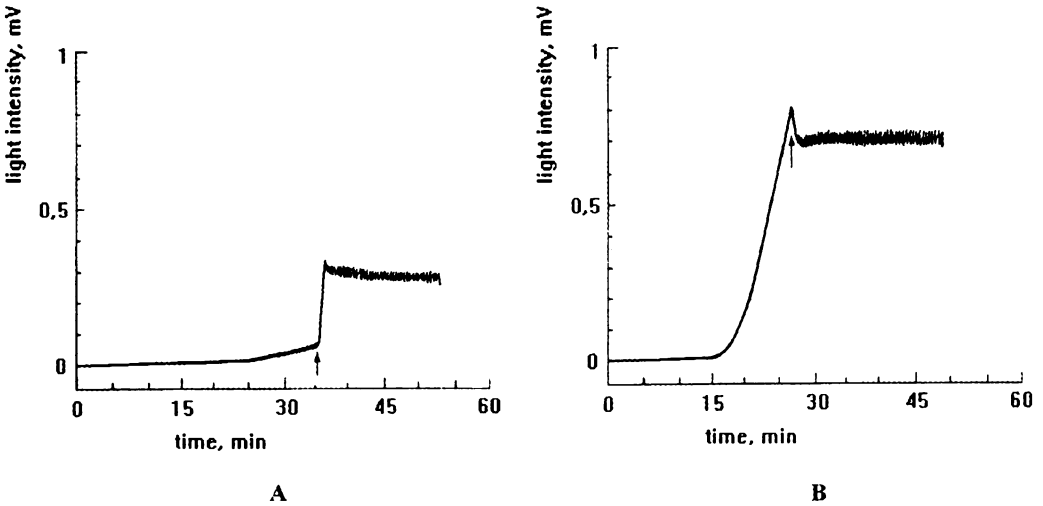

Fig. 7. Time course of the active luciferase accumulation during truncated mRNA translation. (**A**) Luciferase mRNA lack of the last 3′-terminal coding triplet. (**B**) Translation of the full-sized lucRNA. Arrows indicate the time when puromycin was injected into the translation mixtures.

preincubated with RNase H and non-complementary oligonucleotide was translated in the same conditions to rule out any non-specific effect of the oligomer and of RNase H on the translation system. At the indicated time after the start of protein synthesis, 10 mM buffered puromycin solution was added to the translation mixture to give a concentration of 1 mM. Virtually no luciferase activity was observed in the case of truncated RNA (Figure 7A) before addition of puromycin, whereas the typical result of increasing light emission was obtained in the control (Figure 7B). On addition of puromycin the light emission in (A) increased immediately up to a plateau and then stayed constant. The effect of puromycin in the control (B) resembled that of RNase A (see Figure 5). This is not surprising, since puromycin, like RNase A, arrests translation.

Discussion

In previous studies some experimental approaches have been developed to test the tertiary structure of proteins while being synthesized on the ribosomes. These include usage of conformation-specific antibodies (Hamlin and Zabin, 1972; Fedorov *et al.*, 1992), assays for enzymatic activity of growing ribosome-attached polypeptide chains (Zipser and Perrin, 1963; Kiho and Rich, 1964), intrachain disulfide bond formation on nascent polypeptides (Bergman and Kuehl, 1979) and other techniques as reviewed in Gething and Sambrook (1992). The cited studies support the hypothesis that protein folding occurs on the ribosome. However, virtually all of these investigations involved rather long-term procedures for isolating the ribosome and/or the nascent peptides. Furthermore, some of the techniques mentioned above are in fact questionable. For instance, conformation-specific antibodies can, in principle, assist nascent protein folding or even induce the formation of specific tertiary structure. On the other hand, β-galactosidase, the only enzyme shown to be active in the ribosome-bound state, possesses enzymatic activity as a tetramer. This makes any observation of β-galactosidase activity on the ribosome very difficult to interpret. Hence the results of such experiments cannot be regarded as direct evidence for cotranslational folding.

We have used a novel approach to estimate the state of a newly synthesized protein, firefly luciferase, without any preparation step. The cell-free system that we used to translate the lucRNA contained all the necessary components for the luciferase assay (ATP, Mg^{2+} and luciferin). It allowed us to detect the enzymatic activity and thus the folding of enzyme molecules as soon as they are formed in the translation mixture. Both translation and accumulation of the active luciferase stopped within a few seconds after addition of RNase A to the reaction mixture. However, under identical conditions denatured luciferase becomes renatured with a half-time of 14 min. Such a long reactivation time could be explained by the formation of incorrect X-Pro peptide bonds during incubation of the enzyme under denaturing conditions. In most cases the isomerization of these incorrect bonds is the rate-limiting process in the course of protein refolding. Double jump experiments have demonstrated that the rate of luciferase renaturation did not depend on the length of incubation with 8 M urea. This provides evidence that isomerization of X-Pro bonds is not responsible for the slow enzyme reactivation kinetics.

Thus, only a few seconds—if any time—are needed for luciferase to reach its active conformation after it has been released from the ribosome, whereas renaturation from the denatured state characteristically takes at least two orders of magnitude longer. This can be regarded as evidence that luciferase folding occurs during the course of translation and that the virtually completely folded protein is released from ribosomes.

Recently published data support this conclusion: it was shown that the chaperone DnaJ, which appears to be involved in protein folding, binds nascent ribosome-attached polypeptides as short as 77 amino acid residues in the case of firefly luciferase or 55 residues in the case of preprolactin (Hendrick *et al.*, 1993). This indicates that protein folding starts at a very early stage of translation. It seems reasonable to assume that folding of newly synthesized luciferase requires the assistance of appropriate molecular chaperones. An important point is that this process occurs during the synthesis on ribosomes.

The folding of both the newly synthesized luciferase and the denatured one occurred in our experiments in the presence of the complete wheat germ translation system including chaperones. Thus, chaperones could assist protein folding in both cases. It follows that the observed difference in the kinetics of these two processes was caused by factors other than the involvement of chaperones.

We did not detect any luciferase activity while the enzyme was attached to the ribosome, but luciferase, even without the C-terminal amino acid residue, became active immediately upon puromycin addition. Since puromycin is known to release ribosome-bound peptides, the appearance of enzyme activity can be explained as follows. Ribosome-bound luciferase does not catalyze light emission, probably because the 30–40 C-terminal amino acid residues are masked by the ribosome (Malkin and Rich, 1967; Blobel and Sabatini, 1970). This seems quite plausible since the last 12 C-terminal amino acids have previously been shown to be important for luciferase activity (Sala-Newby *et al.*, 1990). Other types of steric interference are also possible. Furthermore, some structural adjustment may be necessary in order for the newly synthesized proteins to attain their final native conformation, as was speculated previously (Tsou, 1988). In our case, luciferase becomes active instantly or very soon after its release by puromycin. Hence, the enzyme appears to be virtually completely folded on the ribosome, even though the ribosome-bound luciferase is inactive, since only a very short time is needed to observe light emission after release.

The results presented above provide evidence that initial folding of firefly luciferase occurs during the course of translation. Further investigations are required to determine the extent and implication of this observation. In any case, cotranslational folding appears to be quite important since newly synthesized protein does not need a prolonged time to become active, in sharp contrast to post-translational folding from the denatured state.

Materials and methods

In vitro transcription of firefly luciferase cDNA

Plasmid pGEM11Zf(−) containing luciferase cDNA under the control of the SP6 promoter was obtained from Promega. The transcription reaction was carried out according to Gurevich *et al.* (1991) in 100 μl (total volume) of 80 mM HEPES–KOH pH 7.5, containing 16 mM $MgCl_2$, 2 mM spermidine, 20 mM dithiothreitol, 3 mM ATP, 3 mM GTP, 3 mM UTP, 3 mM CTP, 2 μl (50 units) of RNasin (Pharmacia), 3 μg of linearized DNA template and 400 units of SP6 RNA polymerase (Fermentas). The reaction was carried out at 37°C for 2 h and stopped by phenol/chloroform extraction. The transcript was purified by G-25 gel filtration and then precipitated by ethanol. An aqueous solution (1 mg/ml) of the transcript was used in translation experiments.

In vitro translation of firefly luciferase

Cell-free translation was performed with a wheat germ extract as described by Hames and Higgins (1984) The reaction mixture contained 20 mM HEPES–KOH pH 7.5, 2 mM $Mg(CH_3COO)_2$, 100 mM KCH_3COO, 2 mM dithiothreitol, 1 mM ATP (buffer A). The final concentration of mRNA was 40 μg/ml and the specific activity of the [^{35}S]methionine was 1 mCi/ml. In some experiments luciferin was added to the translation mixture up to 0.1 mM. The reaction volume was 25 μl and the translation was carried out at 25°C usually for 75 min. To stop protein synthesis quickly, RNase A (from Sigma) was added to the reaction mixture in which translation had proceeded at 25°C. A parallel sample received the same volume of buffer A only.

RNase H digestion of luciferase mRNA

One microgram of full-sized lucRNA was incubated at 37°C with a 50-fold molar excess of complementary 20mer oligodeoxyribonucleotide and 0.5 U RNase H from *Escherichia coli* (Pharmacia). The reaction was carried out for 15 min in the translation mixture without wheat germ extract. The mixture was then returned to a temperature of 25°C. Translation of the resulting truncated mRNA was initiated by addition of wheat germ extract up to 25 μl. As a control, the reaction sequence was carried out with non-specific oligomer (20 nucleotides long).

Luciferase activity assay

Five microliters of the translation mixture were added to 50 μl of 25 mM Tris-CH_3COOH (pH 8.2) containing 5 mM $Mg(CH_3COO)_2$ and 1 mM ATP. The test tube was placed into the luminometer cell at 25°C and 5 μl of 1 mM D-luciferin (Boehringer Mannheim) was injected. The time course of light emission was recorded over time.

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