Free luciferase may acquire a more favorable conformation than ribosome-associated luciferase for its activity expression

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Abstract A variant of firefly luciferase in which the C-terminal end was extended with 44 amino acid residues served as a model protein in this study. After transcription and translation in vitro, the enzyme activity was measured when still attached to the ribosome and when released from the ribosome by incubation with RNase A or puromycin. It was found that the C-terminally extended luciferase already had activity when linked to the ribosome, but its activity was greatly increased when released from the ribosome. These results indicate that the luciferase is folded during synthesis on the ribosome; however, some conformational adjustments occur after its release from the ribosome which are required for the full expression of its enzymatic activity.

Key words: Nascent peptide folding; Luciferase; Enzymatic activity; RNase A; Puromycin

1. Introduction

How nascent peptide chains fold into their native conformations is a basic problem in molecular biology. In 1988, Tsou proposed that peptide segments fold while being synthesized but adjustments are necessary both during and after completion of the entire peptide chain [1]. In recent years, many reports have provided evidence that the nascent peptide begins to fold during its synthesis on the ribosome [2–7]. Luciferase from the North American firefly *Photinus pyralis* catalyzes the ATP and O_2 dependent oxidation of luciferin that results in a green light emission with a peak at 560 nm [8]. As luciferase does not require post-translational processing for enzyme activity, it has served as a model protein for studies of the folding of nascent peptide chains [6,7]. In 1994, Kolb et al. reported that the nascent luciferase peptide showed no activity when still linked to the ribosome, but activity was observed immediately upon its release from the ribosome, while a relatively long time was needed for refolding of the denatured mature luciferase in the same translation system. Therefore they concluded that luciferase begins to fold during its synthesis on the ribosome [6]. This conclusion is supported by the observation that the C-terminal 30–40 amino acid residues of the nascent peptide chain are shielded by the ribosome [9,10] and that removal of the last 12 amino acid residues from the C-terminus of firefly luciferase resulted in the loss of activity [11,12]. In 1996, Makeyev et al. showed that ribosome-associated luciferase acquired enzymatic activity when its C-terminus was extended by at least 26 additional amino acid residues [13]. In 1995, Kudlicki et al. also found that rhodanese extended with 23 amino acids was enzymatically active while bound to the ribosome [14]. These results provide direct support for the hypothesis that proteins acquire tertiary structure as they are formed on the ribosome.

In this study, a luciferase variant, which has the C-terminal end extended with 44 amino acid residues, is used as a model protein to investigate the folding of the protein while it is still linked to the ribosome and after it is released from the ribosome incubated with RNase A or puromycin. Our results show that the luciferase variant is enzymatically active while still bound to the ribosome but that a large increase in activity is observed after it is released from the ribosome, indicating that the protein has folded on the ribosome and that some conformational adjustments may occur upon release from the ribosome.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli strain DH5α was used as a host cell in this study. pTZ18U, which contains multicloning sites and a T_7 promoter, was from Bio-Rad. pGEM-luc, which has a *Bam*HI-*Sal*I restriction fragment bearing the coding region for luciferase, was from Promega. pBVS-1 containing the coding sequence for staphylococcal nuclease R was constructed in this laboratory [15].

2.2. DNA manipulations

Restriction endonucleases, as well as T_4 DNA ligase were used as recommended by the manufacturer (Promega). Plasmid DNA isolation and transformation of $CaCl_2$-treated *E. coli* cells were carried out by the procedure of Sambrook et al. [16]. DNA sequencing was carried out with the T_7 DNA polymerase sequencing system under the conditions described by the manufacturer (Pharmacia).

2.3. Construction of plasmids for expression of luciferase and C-terminally extended luciferase

The plasmid used for expression of the wild-type luciferase in the cell-free translation system in vitro was constructed by isolating the *Bam*HI-*Sal*I restriction fragment using agarose gel electrophoresis after digestion of pGEM-luc with *Bam*HI and *Sal*I. The fragment was then inserted into pTZ18U with the same restriction sites by ligation with T_4 DNA ligase. The recombinant plasmid, named pTZ-LucI, contained the gene encoding the wild-type luciferase which could be expressed under the transcriptional control of the T_7 promoter in the presence of T_7 RNA polymerase.

The plasmid used for expression of the luciferase with C-terminal extension was constructed by amplifying an *Eco*RV-*Kpn*I fragment encoding the C-terminal amino acid residues of luciferase using a standard polymerase chain reaction (PCR) with Taq DNA polymerase in which 5′-TACAAAGGATATCAGGTGG-3′ (*Eco*RV site in the middle) and 5′-CTCGGTACCCAATTTGGACTTTCCGCC-3′ (*Kpn*I tail with a CTC clamp at the 5′ end) oligonucleotides were

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Abbreviation: LucII, luciferase with the extension of 44 amino acid residues

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used as upstream and downstream primers. The reaction substituted the stop codon TAA (UAA) of luciferase with a *Kpn*I recognition site GGTACC encoding glycine and threonine. A string of nucleotides encoding the C-terminal 42 amino acid residues of staphylococcal nuclease R was also amplified by PCR using 5′-CTCGGTACC-GGCTTGTTGGCTAAAGTTGCT-3′ (*Kpn*I tail with a CTC clamp at the 5′ end) and 5′-GGGTCGACGTTAACCTGAATCAGCGT-TGTC-3′ (*Sal*I tail with a GG clamp at the 5′ end and a *Hpa*I site in the middle containing the stop codon TAA) oligonucleotides as upstream and downstream primers. The two PCR products were then ligated with the larger *Eco*RV-*Sal*I fragment of pTZ-lucI after digestion with *Eco*RV/*Kpn*I and *Kpn*I/*Sal*I. In this way, all of the 44 additional amino acid residues were fused in frame with the C-terminal end of luciferase. The recombinant plasmid thus obtained was named pTZ-LucII and the corresponding C-terminally extended luciferase was named LucII (Fig. 1).

2.4. In vitro transcription of the C-terminally extended luciferase gene

The T_7 transcription system (Promega) was used in this experiment. pTZ-LucII was first linearized with *Hpa*I, which can cut off the stop codon TAA (UAA) of the luciferase LucII. The transcription reaction was carried out in 100 μl of transcription mixture containing 4 μg of linearized DNA template and 40 units of T_7 RNA polymerase, incubated at 37°C for 2 h. After transcription, the template DNA was removed by incubation with RNase-free DNase (1 U/μg of linearized DNA template) at 37°C for 15 min. The reaction mixture was further extracted with phenol/chloroform and chloroform and precipitated by ethanol. An aliquot of mRNA solution was purified by G-50 spun-column chromatography to determine the concentration of the mRNA [16]. An aqueous solution of mRNA (2 mg/ml) was used in the translation experiment. The purity of mRNA was examined using agarose gel electrophoresis according to the method of Liu [17].

2.5. In vitro translation of the C-terminally extended luciferase

Cell-free translation was performed with wheat germ extract from Promega in 100 μl. The final concentration of mRNA was 100 μg/ml. The final K^+ concentration was 120 mM and the final Mg^{2+} concentration was 2.1 mM. Cold L-methionine in a final concentration of 80 μM was used in the translation reaction when the products were assayed for luciferase activity. [^{35}S]L-Methionine (1200 Ci/mmol) in a final concentration of 0.5 mCi/ml was used when the product was applied to SDS-PAGE. The translation reaction was carried out at 25°C for 50 min.

2.6. Sucrose gradient centrifugation

A 100 μl aliquot of translation mixture was layered on top of linear 20–45% sucrose gradients in buffer A (20 mM HEPES-KOH pH 7.5, 2 mM $Mg(CH_3COO)_2$, 100 mM KCH_3COO, 2 mM DTT). The mixture was then centrifuged for 2.5 h at 35 000 rpm in an SW40 rotor at 4°C. The gradients were collected from the bottom with approximately 500 μl for each fraction. The absorbency was then measured at 260 nm.

2.7. Assay of luciferase activity

The luciferase assay system from Promega was used to assay the luciferase activity of each gradient fraction. 20 μl of the sample to be assayed was added to 100 μl of the assay buffer. Each reaction mixture was immediately placed in a low level luminescence measurement instrument (model BPCL-4, made in China) with a 560 nm filter to record the time course of light emission. The luciferase activity was related to the light intensity integrated over 60 s.

2.8. Release of nascent peptide from ribosome by incubation with RNase A or puromycin

Two methods were used to release the nascent peptide from the ribosome. To use RNase A to release the nascent peptide from the ribosome, 80 μl of the ribosome fraction was incubated at 10°C with 2 μl of RNase A (10 mg/ml) or distilled water (as control) for 30 min. To use puromycin to release the nascent peptide from the ribosome, 40 μl of the ribosome fraction was incubated at 25°C with 4 μl of puromycin (20 mM) or distilled water (as control) for 30 min in the presence or absence of 2 μl of wheat germ extract or 2 μl of GTP (0.2 mM).

3. Results and discussion

3.1. Construction of the pTZ-LucII containing the gene encoding the C-terminally extended luciferase

The structure of the plasmid pTZ-LucII is shown in Fig. 1. The gene encoding the luciferase LucII (*LucII*) is under the transcriptional control of the T_7 promoter. There is no other ATG between the T_7 promoter and the LucII start codon. Therefore, in the presence of T_7 RNA polymerase, the LucII gene can be correctly transcribed in vitro. There is a unique *Hpa*I site (GTTAAC) at the 3′ end of this gene containing the stop codon TAA (UAA) which can be cut off by incubation with *Hpa*I. The translation of mRNA produced from this *Hpa*I-linearized DNA resulted in ribosome-bound LucII. DNA sequencing analysis indicated that there are two mutant sites (V517G and K541M) in the sequence encoding the C-terminal part of luciferase, but the luciferase activity assay indicated that these mutations do not damage the expression of the luciferase activity.

3.2. Assay of products after transcription and translation in vitro

The extended luciferase mRNA (minus stop codon UAA) was obtained by in vitro transcription as described in Section 2.4. The mRNA is about 1800 nucleotides long and appears as a single band as judged from the 1.2% agarose gel electrophoresis pattern which corresponds well to the *LucII* gene in size (data not shown).

Fig. 2 shows the SDS-PAGE pattern of the proteins after translation. The pattern shows a major protein band with a molecular mass of 66 kDa (lane 1) in contrast to the control (lane 2), indicating that the *LucII* gene has been efficiently transcribed and translated. Some other protein bands of lower molecular mass also appear in lane 1, probably due to premature translation termination in the translation system.

The translation products were fractionated as described in

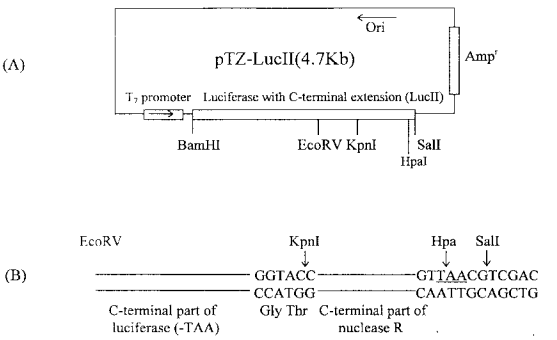

Fig. 1. A: A schematic drawing of pTZ-LucII showing luciferase with C-terminal extension (LucII) under the transcriptional control of the T_7 promoter. The *Eco*RV-*Kpn*I and *Kpn*I-*Sal*I fragments are from the coding sequences of firefly luciferase and the C-terminal part of nuclease R, respectively. The 42 amino acid residues from the C-terminal end of nuclease were fused in frame with the C-terminal end of luciferase through a linker peptide of Gly-Thr by PCR. There is no other ATG between this promoter and the start codon ATG of LucII. The gene can be correctly transcribed in vitro in the presence of T_7 RNA polymerase. B: The partial sequence between the *Eco*RV and *Sal*I sites. The stop codon TAA of luciferase was replaced with the *Kpn*I restriction site (GGTACC, encoding Gly and Thr). There is a unique *Hpa*I site (GTTAAC) at the 3′ end of *LucII* which contains the stop codon TAA. The stop codon can be removed from the *LucII* gene by digestion with *Hpa*I.

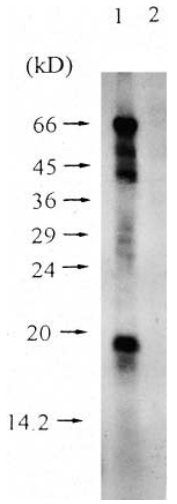

Fig. 2. Fluorogram of SDS gel electrophoresis of the cell-free translation products. The translation products were applied to 15% polyacrylamide gel. The molecular mass in kDa is given on the left. Lane 1, the C-terminally extended luciferase translated from the mRNA transcribed from pTZ-LucII linearized with *Hpa*I. Lane 2, the translation products without the added mRNA.

Section 2.6. As shown in Fig. 3, two luciferase activity peaks were detected corresponding to the 80S ribosome fraction and the free protein fraction of the translation products. The activity in the free protein fraction means that there are large amounts of full length luciferase being released from the ribosome, although the stop codon is removed from the mRNA, as was observed previously [13].

3.3. *Folding of the nascent luciferase is a cotranslational process*

As shown above, luciferase activity was detected in the 80S ribosome fraction, indicating that the luciferase LucII has already folded into an enzymatically active conformation while still linked to the ribosome. This result suggests that the folding of the nascent peptide of LucII is a cotranslational process, which is consistent with Makeyev [13].

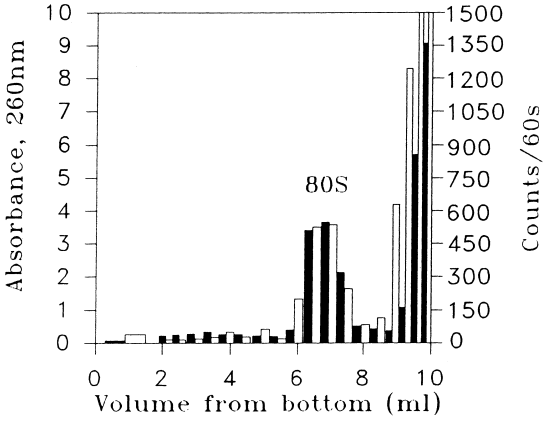

Fig. 3. Nascent luciferase activity in the 80S ribosome fraction of the wheat germ translation system. Cell-free translation products of luciferase LucII were fractionated as described in the text. The luciferase activity in sucrose gradient fractions is shown by the unfilled columns. The absorbance at 260 nm is shown by the filled columns.

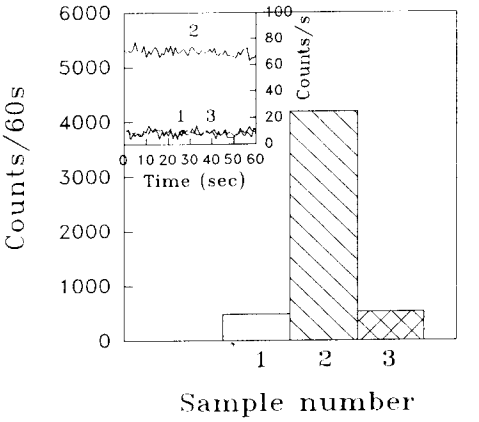

Fig. 4. Activity of luciferase LucII in the 80S ribosome fraction before and after treatment with RNase A. The ribosome fractions were treated with distilled water (1) or RNase A (2) or were not treated (3). The inset shows the original experimental data.

3.4. *Free luciferase may acquire a more favorable conformation for activity expression than ribosome-associated luciferase*

The luciferase activity in the 80S ribosome fractions before release from the ribosome was compared with the activity after release. As shown in Fig. 4, the luciferase activity in the ribosome fraction treated with RNase A (sample 2) is about 8.7 times that of the ribosome fraction without RNase A treatment (samples 1 and 3). Although some other possibilities exist, the most reasonable explanation for the activity increase is that the luciferase molecule acquires a more favorable conformation state for expression of its activity after release from the ribosome due to changes in its microenvironment, suggesting that some structural adjustment may be necessary for the newly synthesized proteins to attain their final conformation, as was speculated previously [1,6].

Puromycin usually acts as an aminoacyl-tRNA analogue to release the nascent peptide from the ribosome [13,14]. In this experiment, puromycin was used to release LucII from the ribosome. As shown in Fig. 5, addition of puromycin (sample 2) resulted in a slight activity increase for the ribosome-associated LucII, but its efficiency was much lower than that of RNase A. Addition of both puromycin and wheat germ ex-

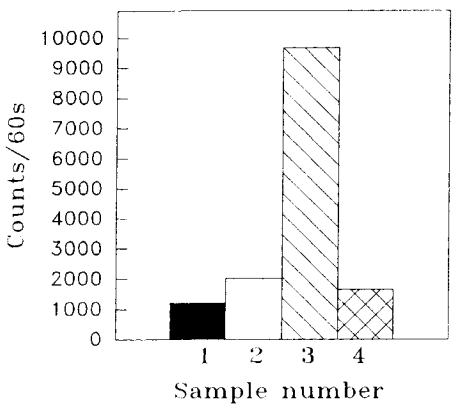

Fig. 5. Activity of luciferase LucII in the 80S ribosome fraction before and after treatment with puromycin. The ribosome fractions were treated with distilled water (1), puromycin (2) or puromycin and wheat germ extract (3) or were not treated (4).

tract (sample 3) resulted in a large activity increase, whereas puromycin mixed with GTP did not cause as large an activity increase (data not shown), indicating that some component other than GTP could promote the puromycin-induced release of the ribosome-associated LucII.

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