Enzymatic activity of the ribosome-bound nascent polypeptide

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Abstract Firefly luciferase was shown to be completely folded and thus enzymatically active immediately upon release from the ribosome [Kolb et al. (1994) EMBO J. 13, 3631–3637]. However, no luciferase activity was observed while full-length luciferase was attached to the ribosome as a peptidyl-tRNA, probably because the C-terminal portion of the enzyme is masked by the ribosome and/or ribosome-associated proteins. Here we have demonstrated that the ribosome-bound enzyme acquires the enzymatic activity when its C-terminus is extended by at least 26 additional amino acid residues. The results demonstrate that the acquisition of the final native conformation by a nascent protein does not need the release of the protein from the ribosome.

Key words: Nascent peptide; Enzymatic activity; Luciferase

1. Introduction

The light-producing enzyme, luciferase of the firefly *Photinus pyralis*, has been the subject of recent studies on protein folding [1–6]. This enzyme has been shown to start the folding cotranslationally [1,2]. At the same time, the full-length enzyme is virtually inactive in the ribosome-bound state, although the acquisition of the activity occurs immediately upon release from the ribosome [1]. The most plausible speculation as regards this phenomenon is that the C-terminal part of a nascent polypeptide as long as 30 to 40 residues is shielded by the ribosome in the so-called ribosomal tunnel [7–11] or/and by some nascent peptide-associated protein(s) [2–6]. This seems to be particularly realistic in the case of firefly luciferase: its activity decreases dramatically with the removal of its last 12 C-terminal amino acids [12] which probably participate in the formation of the enzyme active center. On the other hand there is evidence that firefly luciferase nascent chains interact with a set of chaperones during synthesis in a mammalian cell-free translation system [2,4–6]. According to current concepts the release of nascent chain from the ribosome is followed by its interaction with chaperones that promotes protein folding [2,14,15].

The question arises whether the release of the full-length nascent luciferase is requisite to attain the final native structure, or can the enzyme be active on the ribosome. To answer this question several recombinant luciferases were constructed. All luciferase gene constructs lacked stop codons and thus produced, upon in vitro expression, full-length luciferase polypeptide extended by 1 to 59 amino acid residues at the C-terminus and still attached to the ribosome. The ribosomes bearing those elongated luciferases were isolated and the enzyme activity assay revealed that the nascent luciferase was enzymatically active if the C-terminus has been extended by 26, 27, 31, 42 and 59 extra amino acid residues. Extensions by 1, 12 or 21 residues were not enough for the ribosome-bound nascent enzyme to attain its active conformation.

These results were first reported at the conference 'Frontiers in Translation', Victoria, B.C., Canada, May 20–25, 1995 (see [16]). The principal result was reproduced by another group with another enzyme, rhodanese [17].

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Abbreviations: aa, amino acid residue; luc, luciferase; NPT II, neomycin phosphotransferase; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate.

2. Materials and methods

2.1. Plasmids

The pGEM luc(−stop) plasmid was derived from pGEM luc (Promega) by the following way: the *Nde*I restriction site was introduced into pGEM luc in place of the luciferase stop codon by oligonucleotide-directed mutagenesis resulting in substitution of the original 5′TAA AATG-3′ with 5′-TCA TATG-3′. Thus, the natural UAA stop codon of luciferase was substituted by UCA, a serine coding triplet. Translation of mRNAs produced from this construct resulted in ribosome-bound luciferase extended by an unnatural (random) amino acid sequence.

The pTZ luc(−stop) plasmid was constructed by subcloning *Hin*dIII/*Sac*I fragment of pGEM luc(−stop) containing luciferase gene into the pTZ 19R vector.

To construct pTZ luc(NPT II) plasmid the transposon tn5 neomycin phosphotransferase (NPT II) was used. The NPT II coding region, lacking the two N-terminal triplets, was amplified by 30 cycles of standard PCR with Tth DNA polymerase. 5′-TTCCATATGAACAAGATGGATTGC-3′ and 5′-CTTCATATGCCCAGAGTCCCG-3′ oligonucleotides were used as upstream and downstream primers, respectively. Both primers carry *Nde*I sites at their 5′-ends. After digestion of the PCR product with *Nde*I it was inserted at the *Nde*I site of pTZ luc(−stop). Transcription and translation of the resulting pTZ luc(NPT II) template produced the recombinant protein where the complete luciferase amino acid sequence was followed by NPT II with Ser Tyr in place of the naturally occurring N-terminal Met Ile.

2.2. Preparation of mRNAs

Messenger RNAs were prepared by in vitro SP6 or T7 transcription [18] using pGEM luc(−stop) (Fig. 1A) or pTZ luc(NPT II) (Fig. 1B), respectively. In order to produce mRNAs with different extensions the DNA templates were cut by appropriate restriction endonucleases. Preparation of mRNA for luciferase extended by NPT II was done by transcription of PCR-generated fragments containing T7 promoter, luciferase coding region and N-terminal portion of the NPT II gene (Fig. 1B). PCR was carried out with the universal T7 upstream primer and one of the four downstream primers producing luciferase extended by 12, 19, 27 or 42 coding triplets. The plasmid pTZ luc(NPT II) was linearized with *Pst*I and then amplified using Tth DNA polymerase by 30 cycles of PCR consisted of 1 min at 94°C, 1 min at 50°C and 2.5 min at 72°C plus 4 s extention on each cycle. In each case three independent experiments were performed to rule out any effect due to possible mutations caused by Tth DNA polymerase.

2.3. Cell-free translation

Cell-free translation was performed with a wheat germ extract as

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described in [1]. The reaction volume was 100 μl and the translation was carried out at 25°C for 40 min. The specific activity of the [^{35}S]methionine was 1 mCi/ml. There was no luciferin addition to the translation mixture.

2.4. Sucrose gradient centrifugation

After incubation at 25°C 100 μl aliquot of cell-free translation system was diluted with equal volume of ice-cold buffer A (20 mM HEPES-KOH pH 7.5, 2 mM $Mg(CH_3COO)_2$, 100 mM KCH_3COO, 2 mM dithiothreitol) and layered on the top of linear 0.5 to 1.5 M sucrose gradients in buffer A. Centrifugation was carried out for 2 h at 40,000 rpm in an SW41 rotor at + 4°C. The gradients were pumped from the bottom and the absorbance at 279 nm was continuously recorded. 630 μl fractions were collected at + 4°C.

2.5. Luciferase activity assay and puromycin reaction

180 μl aliquot from each gradient fraction was immediately assayed for luciferase activity by mixing with 20 ml of solution containing 5 mM ATP and 5 mM luciferin in the luminometer. For puromycin reaction an aliquot of translation system or sucrose gradient fraction was incubated for 5 min at 25°C in the presence of 0.5 mM puromycin.

2.6. Miscellaneous

Restriction endonucleases, *Stu*I, *Xho*I, *Ecl*136I, *Pst*I, *Hin*dIII and *Sac*I were from Fermentas. *Nde*I and *Bal*I as well as T4 DNA ligase were purchased from Promega. Tth DNA polymerase was isolated and purified from *Thermus thermophilus* in our laboratory. Other chemicals were from Sigma. SDS electrophoresis was carried out in polyacrylamide gel according to Laemmli [19].

3. Results and discussion

3.1. Synthesis of extended luciferase in wheat germ translation system

Two types of extension were employed to synthesize the ribosome-bound luciferase with extended C-terminus. In the first case polypeptide sequence of the unnatural origin (obtained in the course of the plasmid polylinker transcription and subsequent translation) was added to the luciferase C-terminus. In the second case the N-terminal portion of neomycin phosphotransferase II was fused to the luciferase sequence to extend the enzyme by the naturally occurring sequence. DNA templates for transcription were prepared using different restriction sites and PCR primers to produce different extension lengths from 1 to 59 codons (described in Fig. 1). The extended luciferase mRNAs were obtained by either SP6 or T7 transcription of these chimerical DNA templates. Cell-free translation of the transcripts was performed in the wheat germ extract as described in [1]. All of the full-sized translation products were of the expected size as judged from the corresponding SDS-electrophoretic patterns (Fig. 1).

3.2. Enzymatic activity of ribosome-bound extended luciferase

The translation reactions were fractionated by sucrose gradi-

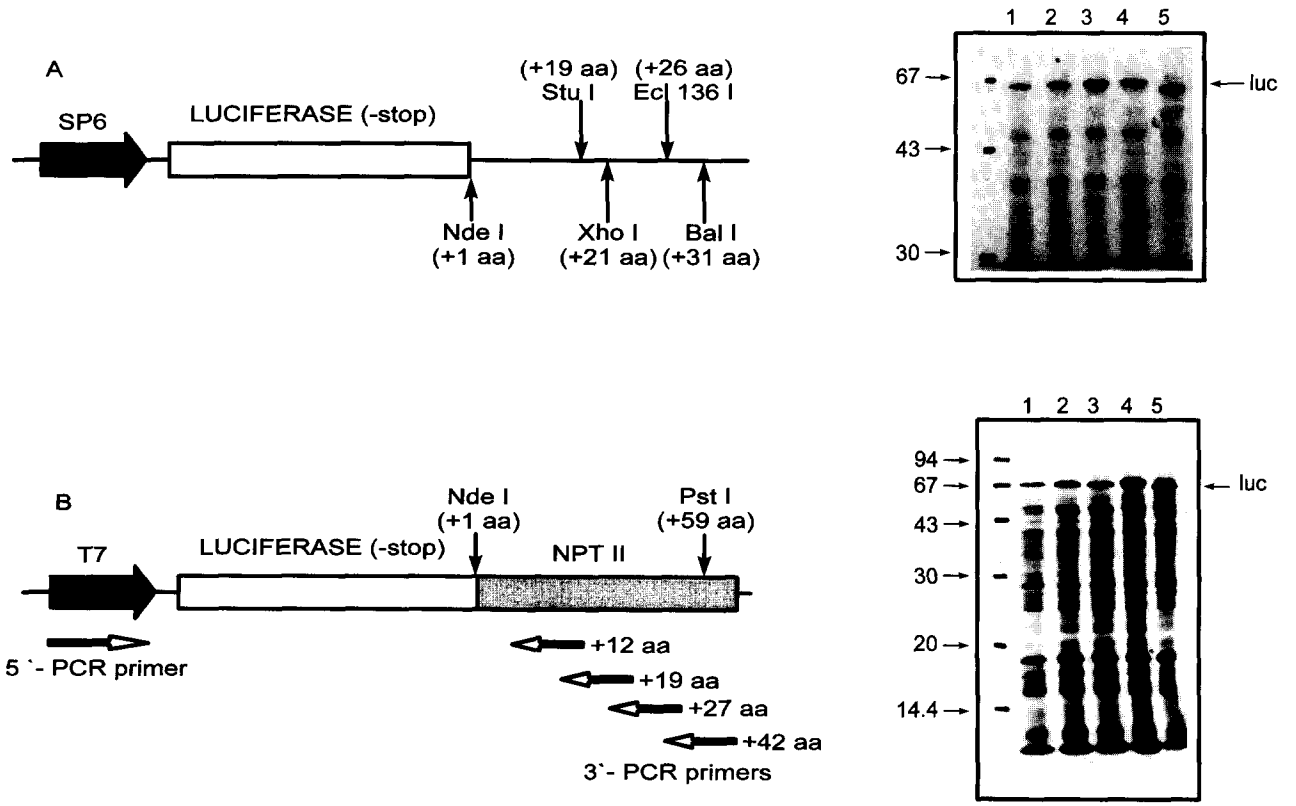

Fig. 1. DNA templates for synthesis of extended luciferases and autoradiograms of SDS gel-electrophoresis of the cell-free translation products. Restriction sites are shown by vertical arrows, corresponding extension lengths in amino acid residues (aa) are given in parentheses. Horizontal arrows point the position of molecular weight standards on the autoradiogram of the gel. Molecular mass in kDa is given to the left of the arrows. The position of unextended luciferase is shown by 'luc'. (A) pGEM luc(−stop) plasmid. Electrophoresis was carried out in 10% polyacrylamide gel. Line 1, extension with 19 aa; lane 2, extension with 21 aa; lane 3, extension with 26 aa; lane 4, extension with 31 aa; lane 5, extension with 1 aa. (B) pTZ luc(NPT II) plasmid. Lengths of extension obtained by PCR with subsequent transcription and translation are shown to the right of 3′-PCR primers. Electrophoresis was carried out in 15% polyacrylamide gel. Line 1, extension with 12 aa; lane 2, extension with 19 aa; lane 3, extension with 27 aa; lane 4, extension with 59 aa; lane 5, extension with 1 aa.

ent centrifugation. Luciferase activity assay in the gradient fractions revealed that nascent luciferase was enzymatically active if the C-terminus was extended by 27, or 42, or 59 N-terminal amino acid residues of neomycin phosphotransferase II (Fig. 2). Extensions by 1, 12 or 19 residues were not enough for the ribosome-bound nascent enzyme to attain its active conformation. Such an activation of nascent luciferase by the extension of its C-terminal portion did not depend on the polypeptide sequence of the extension. Elongation of the enzyme by the sequence of unnatural origin (plasmid polylinker) led to a similar result: 26 or 31 amino acid residues of extension allowed to acquire the functional structure of ribosome-bound luciferase, while 1, 19, or 21 residues did not (Fig. 3). Luciferase activities measured in the 80S fractions of sucrose gradients are summarized in Fig. 4.

Puromycin reaction was used to test whether extended luciferase was bound to the ribosome as peptidyl-tRNA or there was a nonspecific binding. The test was carried out with luciferase that was active in the ribosome-bound state due to extension by 27 residues. The addition of puromycin to the translation system before fractionation revealed the absence of luciferase activity in the gradient fractions corresponding tot he 80S ribosome peak (Fig. 5A and B). The activity was only found at the top of the gradient. This proves that luciferase was bound to the ribosome as puromycin-sensitive peptidyl-tRNA.

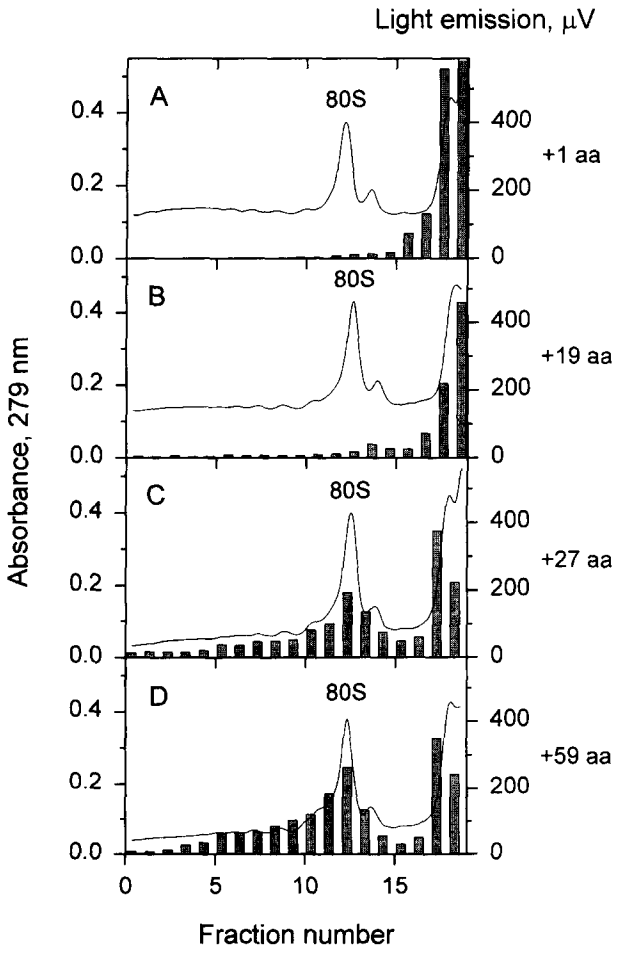

Fig. 2. Nascent luciferase activity in the 80S ribosome fraction of wheat germ translation system. Luciferase C-terminal portion are extended by addition of 1 (A), 19 (B), 27 (C) and 59 (D) amino acid residues of NPT II N-terminal fragment. Light emission intensity in sucrose gradient fractions is shown by bars.

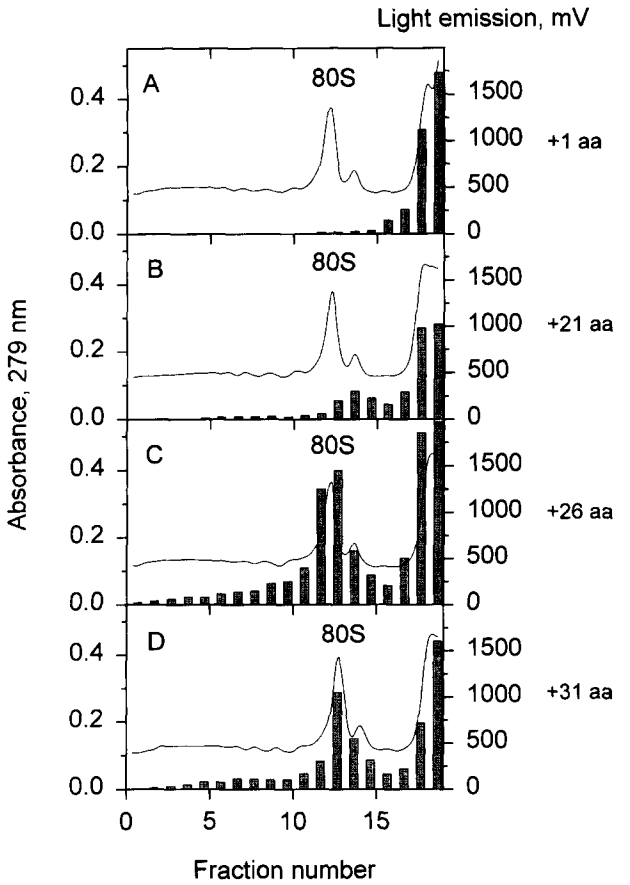

Fig. 3. Nascent luciferase activity in the 80S ribosome fraction of wheat germ translation system. Luciferase C-terminal portion are extended by addition of 1 (A), 21 (B), 26 (C) and 31 (D) amino acid residues of unnatural polypeptide, obtained in the course of translation of the plasmid polylinker sequence. Light emission intensity in sucrose gradient fractions is shown by bars.

The enzyme that was elongated by 19 amino acid residues and was inactive on the ribosome became active upon puromycin-induced release from the ribosome (compare Fig. 5C and D), so luciferase was not damaged by the addition of extra residues to its C-terminus.

The previous observations of the enzymatic [20–23] or immunological [24] activity of growing ribosome-bound β-galactosidase can be explained by the presence of the free enzyme subunits associated with the nascent chain, since β-galactosidase is known to be active as a tetramer. In contrast, the firefly luciferase is active as monomer. Thus, our experiments clearly demonstrate that the final native structure and biological activity can be attained by a single nascent polypeptide on the ribosome, without its previous release.

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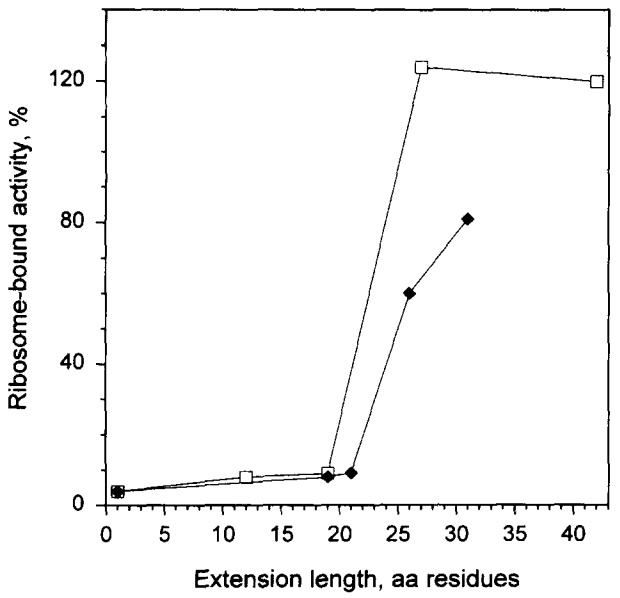

Fig. 4. Enzymatic activity of the ribosome-bound nascent luciferase as a function of C-terminal extension length. One hundred percent corresponds to the activity measured in the ribosome fraction after puromycin-induced release of the enzyme. The open symbols show the activity of luciferase extended with NPT II, the closed symbols belong to luciferase extended with random polypeptide.

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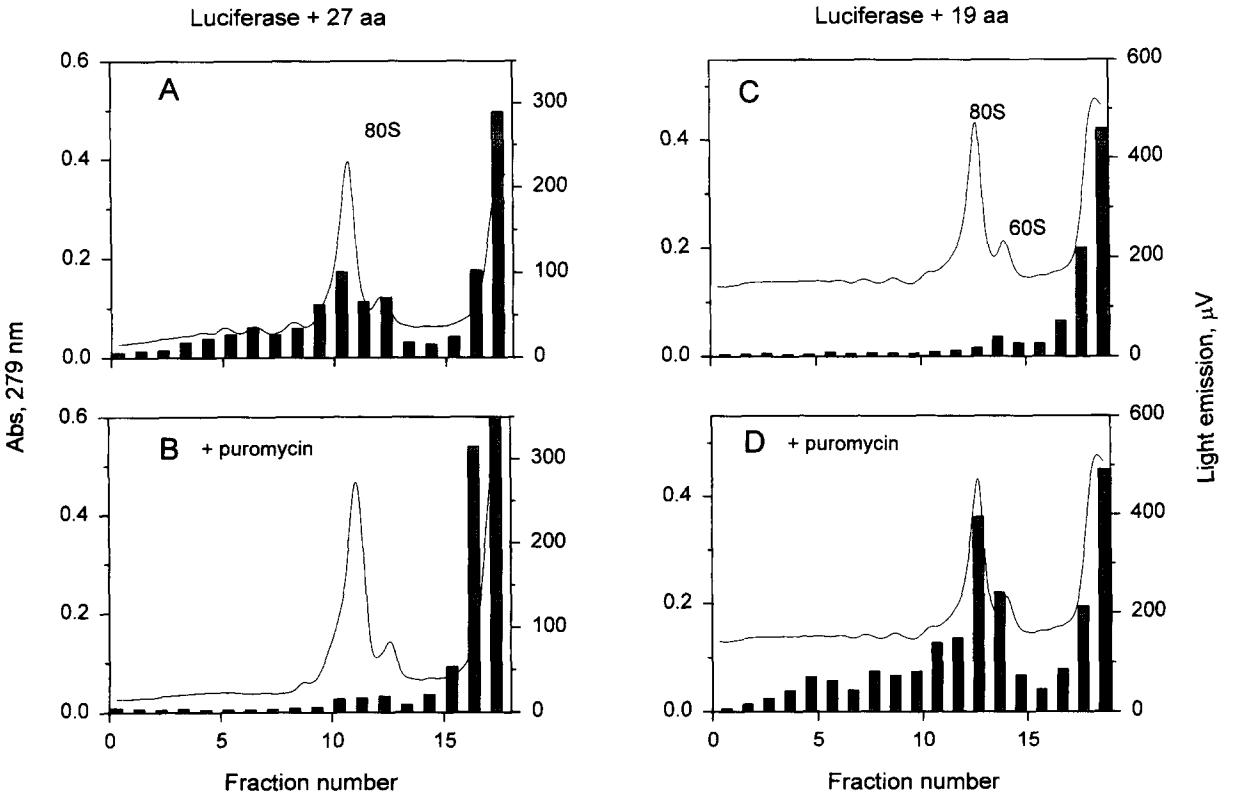

Fig. 5. Puromycin-induced release of luciferase activity from the ribosome. (A) Activity of luciferase extended with 27 amino acid residues of NPT II in sucrose gradient fractions. The enzyme is active in the ribosome-bound state. (B) Puromycin is added to the sample A before centrifugation. The activity is released from 80S fraction. (C) Activity of luciferase extended with 19 amino acid residues of NPT II in sucrose gradient fractions. the enzyme is inactive on the ribosome. (D) Puromycin is added to each fraction of gradient C after centrifugation. Luciferase is released from the ribosome and becomes active.

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