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Force measurements of the disruption of the nascent polypeptide chain from the ribosome by optical tweezers

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ABSTRACT

We show that optical tweezers are a valuable tool to study the co-translational folding of a nascent polypeptide chain at the ribosome in real-time. The aim of this study was to demonstrate that a stable and intact population of ribosomes can be tethered to polystyrene beads and that specific hook-ups to the nascent polypeptide chain by dsDNA handles, immobilized on a second bead, can be detected. A rupture force of the nascent chain in the range of 10–50 pN was measured, which demonstrates that the system is anchored to the surface in a stable and specific way. This will allow in numerous future applications to follow protein folding using much lower forces.

1. Introduction

Despite tremendous efforts and remarkable progress in the understanding of thermodynamics and kinetics of protein folding processes in vitro (refolding following unfolding), our knowledge of protein folding in cells is rather limited. Since it is obvious that ineffective folding or misfolding could be the basis for various diseases, more and more questions arise that need to be addressed. Accumulation of insoluble protein aggregates is an often observed phenomenon during aging of organisms and occurs in many age-related neurodegenerative diseases. A deeper insight into the mechanisms of protein folding in vivo is therefore highly desirable but still difficult to achieve. To approach protein folding in the cellular context we propose to investigate co-translational folding in a cell free transcription/translation system, using single-molecule manipulation techniques. Optical tweezers have been used to monitor the RNA–tRNA interaction and the translational kinetics of the RNA within the molecular machinery of the ribosome [1]. When using optical tweezers the variations of the forces during the synthesis of the nascent polypeptide chain at the ribosome can be monitored. In a co-translational folding experiment the time scale of folding is largely determined by the rate of synthesis of the nascent chain in contrast to in vitro refolding [2]. The relevant questions are: (i) What are the new folding intermediates in an in vivo experiment? (ii) What are the different folding pathways which are otherwise hidden in an ensemble experiment where only a mean value of a great number of non-synchronous processes is extracted?

Co-translational folding of proteins has been studied in the past. Thereby the protein remains bound on the ribosome via a C-terminal extension of at least 30 aminoacids, which spans the full length of the ribosomal tunnel, thus giving the protein the possibility to fold completely [2–4]. Ensemble measurements of these molecules give a mean value of an asynchronous process like protein folding taking place in the bulk. Single molecule studies, on the other hand, give valuable information about asynchronous processes and evidence for reaction intermediates by eliminating the temporal and population averaging characteristics of bulk ensembles [5,6].

However, two very elegant folding studies should be mentioned where folding intermediates can be observed from ensemble measurements. In these investigations a cell free transcription/translation system produces polypeptide chains of different length

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starting synthesis at the wild type N-terminus. In one of these studies the folding of the polypeptides of different length was investigated by spectroscopic methods. A non-native β-sheet conformation detected at short length converts into the native α-helical structure with increasing chain length [7,8]. In a second study also using a transcription/translation system the nascent chain stayed on the ribosome and the structure was investigated by high-resolution NMR. In this case selective ^{13}C and ^{15}N labeling of the chains was performed by the cell free system. The great advantage of this method is that high resolution structures of folding intermediates can be obtained [9,10].

Single molecule fluorescence studies on proteins in cells gave some impressive results [11,12], but the manipulation of the sample is often quite difficult and the fluorescent dyes that can be used under these conditions are rather limited and with inferior photophysical properties. In vitro cell-free single molecule fluorescent studies, where translating ribosomes are immobilized on a surface provide a deeper insight into co-translational protein synthesis and folding [13–15].

In this study, we report the use of translating ribosomes immobilized on a polystyrene bead to measure the forces applied on the nascent polypeptide chain during co-translational protein synthesis with the use of optical tweezers.

2. Materials and methods

2.1. Biotinylation of ribosomes in vivo

Strain AVB101 (Avidity, Denver, CL) is an *Escherichia coli* B strain that contains a pACYC184 plasmid with a birA gene to overexpress biotin ligase (pBirAcm). L4 protein was cloned to the biotinylation vector pAN5 (Avidity, Denver, USA) behind the coding sequence of the N-terminal AviTag which can be biotinylated in vivo by the biotin ligase and transformed into AVB101. Expression of both biotin ligase and the AviTag-L4 fusion was induced with IPTG (1 mM) at A_{595} = 0.6 for 3 h. Biotin was also added at the time of induction to a concentration of 50 μM.

2.2. Isolation of ribosomes

Biotinylated ribosomes were isolated [16] and resuspended in Tico buffer [20 mM Hepes/KOH (pH 7.6 at 0 °C), 6 mM magnesium acetate, 30 mM ammonium acetate, 4 mM β-mercaptoethanol]. They were probed for activity with poly(U)-dependent poly(Phe) synthesis [17] and with FABP (Fatty Acid Binding Protein) synthesis in an in vitro transcription/translation system. In both cases, the produced polypeptide was precipitated with TCA 5% and the amount of ^{14}C-Phe and ^{14}C-Leu, respectively, was measured in a scintillation counter.

2.3. Plasmid construction

To the plasmid pRSET /EmGFP (Invitrogen) which contains the gene for GFP Emerald (GFPem: S65T, S72A, N149 K, M153T, I167T) we added the coding region for 31 additional aminoacids downstream of the GFP gene between the XhoI site and the HindIII site. For the amplification of the elongated GFP gene by PCR the primers FP1 (5′-GCGGATCCGAATTCGCCACCATGGTGAGC-AAGGGCGAGGAGC-3′) (NcoI site is underlined) and RP1 (5′-GCTTA-AAGCTTCGCCTTCTCGTTCAGTAAAGTCTTAACGCGTGCGACATC GCGACGCACGCCTACACGGTCAGCCTTGCCGGTTGCCGCATTCTCGAG CTTGTACAGCTC GTCCATGCCGAG-3′) (HindIII site is underlined and XhoI site is double underlined) were used. Primer FP1 includes the NcoI site containing the start codon of GFPem and primer RP1 includes the coding region for the additional 31 aminoacids (LENA-ATGKADRVGVRRDVARVKTLLNEKAKL) and lacks a stop codon. The PCR product (~800 bp) was exchanged against the fragment between the NcoI and HindIII sites of the original vector. The resulting plasmid was further modified by inserting the amber stop codon immediately after the NdeI site and a sequence of 6 histidines (6xHis) before the NcoI site. Primers FP2 (5′-ATACATATGTAG GGTGT GGCTAGCGTGA CTGGTGGACAG-3′) (NdeI site is underlined and amber stop codon is double underlined) and RP2 (5′-GCC ATGGTGTGATGGTGATGGTGATGGCGAATTCGGATCCCCATCGATC-3′) (NcoI site is underlined and 6xHis sequence is double underlined) were used giving a fragment of ~150 bp. Primer FP2 includes the NdeI site containing the start codon of the construct and immediately after that the amber stop codon, where a biotinylated lysine was incorporated during synthesis using the suppressor tRNA technique [18–20]. Primer RP2 includes the sequence of 6xHis that was used to halt synthesis. The final plasmid was linearized by digesting with HindIII and was dephosphorylated.

2.4. Coupling of DNA handles to polystyrene beads

DNA molecules were prepared by PCR amplification of the pTYB1 plasmid (7477 bp) [New England Biolabs (NEB)] using either 5′-Thiol or 5′-DIG and 5′-Biotin end-modified primers. The 4056-bp PCR fragment was then coupled to anti-DIG beads. These beads were prepared by cross-linking anti-digoxigenin (Roche) to Protein G beads (Spherotech).

2.5. In vitro transcription/translation and incorporation of biotin during synthesis

Polystyrene beads (3.1–3.9 μm in diameter) covered with streptavidin (Spherotech) were incubated with 20 times excess of biotinylated ribosomes in Tico buffer and they were allowed to react overnight at 4 °C. Beads were centrifuged down and non-bound ribosomes were removed. The unreacted streptavidin molecules on the bead were blocked with 300 μM of biotin for 1 h on ice. GFPem was synthesized by the tethered ribosomes using an in vitro transcription/translation fractionated system (RiNA GmbH, Berlin, Germany). 5.5 nM linearized plasmid containing the target gene were used and 5 μM of an oligonucleotide having the antisense sequence of tmRNA (5′-TTAAGCTGCTAAAGCGTA GTTTT-CGTCGTTTGCGACTA-3′) was added in order to inhibit tmRNA assisted release [21,22]. The synthesis was carried out for 30 min at room temperature (~25 °C) as described before [14]. GFP was biotinylated at the N-terminus during synthesis using the suppressor tRNA technique [18]. Briefly, a modified $tRNA^{Lys}$, which carries a biotinylated lysine and recognizes the amber stop codon was added to the transcription/translation system at a final concentration of 10 μM. During synthesis the amber stop codon was suppressed by the suppressor tRNA and instead of protein release the biotin was inserted in that position. Additionally the transcription/translation system was lacking the aminoacid histidine, thus synthesis was halted when it reached the sequence for 6xHis. Finally the mix was incubated with 100 mg/ml streptavidin for 1 h on ice, centrifuged down and unreacted excess of streptavidin was removed.

2.6. Optical tweezers setup

Experiments were performed on a dual-beam optical tweezers instrument [23], which also has fluorescence detection capabilities. The bead-to-bead end distance was measured from both the movement of the micropipette (controlled with a closed-loop piezoelectric element) and the deflection of the laser. The pipette bead was moved away from the trapped bead at a constant velocity of about 1 nm/ms. All signals (distance, force) were low-pass filtered at

159 Hz. Force curves experiments were performed at room temperature.

3. Results and discussion

A lot of different techniques were used in the past for the immobilization of the active translation machinery. Initially physical adsorption [24] or non-specific covalent immobilization through surface exposed cysteine residues [25], then either through biotinylated mRNA making use of the specific biotin–streptavidin interaction [26,27] or by modifying the rRNA for a digoxigenin/anti-digoxigenin coupling [13,28] were applied. In this study, we used the biotin–streptavidin interaction by in vivo biotinylating the ribosomal protein L4, a technique we also employed before to tether ribosomes on PEG-coated surfaces [14] (see Methods online).

L4 biotinylated ribosomes were specifically attached on micron-sized polystyrene beads (3.1–3.9 μm in diameter) coated with streptavidin (Fig. 1A). The streptavidin on the bead that did not react with ribosomes was blocked by adding 650 μM d-biotin in 10 mM bicine buffer. By using a fractionated cell free transcription/translation *E. coli* system, tethered ribosomes started to synthesize GFP. The GFP construct was extended at the C-terminal end by a sequence of 31 additional aminoacids in order to give the full length protein the possibility to fold correctly outside the ribosomal tunnel [29] and protein release was suppressed to keep GFP bound on the ribosome after synthesis (Fig. 1B).

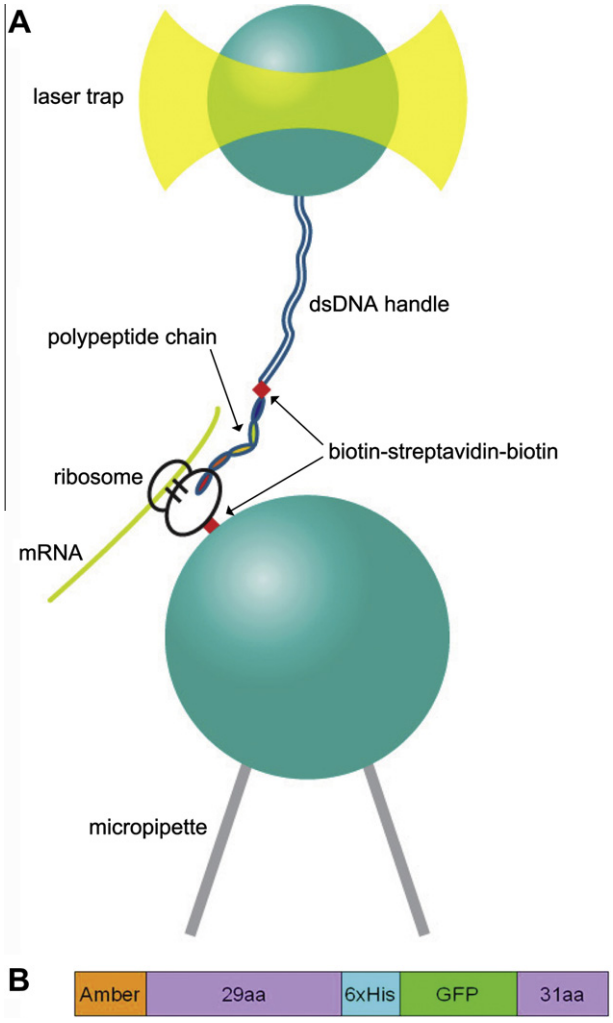

Fig. 1. Schematic presentation of the setup inside the flow chamber and the construct used. (A) Ribosomes attached on one bead via biotin–streptavidin interactions synthesize a polypeptide chain. Synthesis is halted when the polypeptide chain appears outside the ribosomal tunnel. A DNA handle attached on a second bead, which is trapped by the optical tweezers, is able to catch the N-terminus of the polypeptide chain via biotin–streptavidin interactions. (B) Schematic presentation of the DNA construct used in the transcription/translation system.

In order to introduce a biotin to the N-terminus of the nascent polypeptide chain, an amber stop codon was inserted followed by a linker sequence of 29 aminoacids plus a sequence of 6 histidines (6xHis) (Fig. 1B). A modified $tRNA^{Lys}$ carrying a biotinylated lysine and able to recognize and pair with the amber stop codon was also added to the transcription/translation system. According to the suppressor tRNA technique, the biotinylated lysine was incorporated during synthesis at the N-terminus of the construct [18–20]. In order to stop the synthesis at a desired position we used a histidine deficient transcription/translation system. Thus, the synthesis of GFP proceeded until it reached the 6xHis and halted, at which point the biotinylated N-terminus was just appearing outside of the ribosomal tunnel. In order to prove this, we incubated the above construct, as well as control ribosomes that were not synthesizing, with a cy5-labeled streptavidin. Observation with a confocal microscope revealed fluorescently labeled beads in the first case, but not in the control, suggesting that the biotinylated nascent chain was accessible and could bind to streptavidin. Following this pre-synthesis, ribosomes were incubated with streptavidin yielding a streptavidin–biotin N-terminus of GFP.

We injected the beads, with streptavidin on the N-terminus appearing outside of the tethered ribosomes, into a custom-made flow chamber [23]. A single bead was trapped by the tweezers and was then transferred to a micropipette (<1 μm diameter) connected to a manual syringe by applying aspiration. The chamber was attached to a piezoelectric stage with a maximum extension of 100 μm for the *X* and *Y* direction and 20 μm for the *Z*-axis and could move accurately in nm steps [23].

As a control we restarted the paused synthesis of GFP by adding histidine to the transcription/translation system, in order to check the activity of the translation machinery on the beads. Fluorescence of single GFP molecules was observed on the surface of beads immobilized on the micropipette (see online video in Supplement).

While holding on the micropipette a single bead with translating ribosomes halted at the 6xHis, we injected into the chamber a second set of polystyrene beads to which dsDNA was attached (see below) to trap one of them in the optical tweezers. The dsDNA molecules were attached from one end to the surface of the beads through a digoxigenin/anti-digoxigenin (DIG/anti-DIG) coupling or a covalent coupling through thiol, whereas the free ends were biotinylated and could interact with the streptavidin of the nascent polypeptide chain. The DNA handles thereby act also as spacers to limit the non-specific interactions between the two beads [30] (Fig. 1A). The length of the constructs produced by PCR are 4056 bp (1216 nm) and 5624 bp (1874 nm).

By approaching the micropipette close enough to the bead in the optical trap a force was measured by the detector. In order to determine if the coupling was specific we pulled the micropipette slowly away from the optical trap and we monitored the applied force versus the extension up to the point where a rupture occurred. In most measurements the rupture was observed in the range of 10–50 pN (Fig. 2A and B), which is consistent with the force that is required to break the hydrogen bonding between the mRNA and the peptidyl-tRNA carrying the nascent chain (codon–anticodon interactions) [28]. This indicates that the biotinylated end of the dsDNA interacted specifically with the streptavidin at the N-terminus of the paused nascent chain. At some point when the force became too large and the hydrogen bonding between the

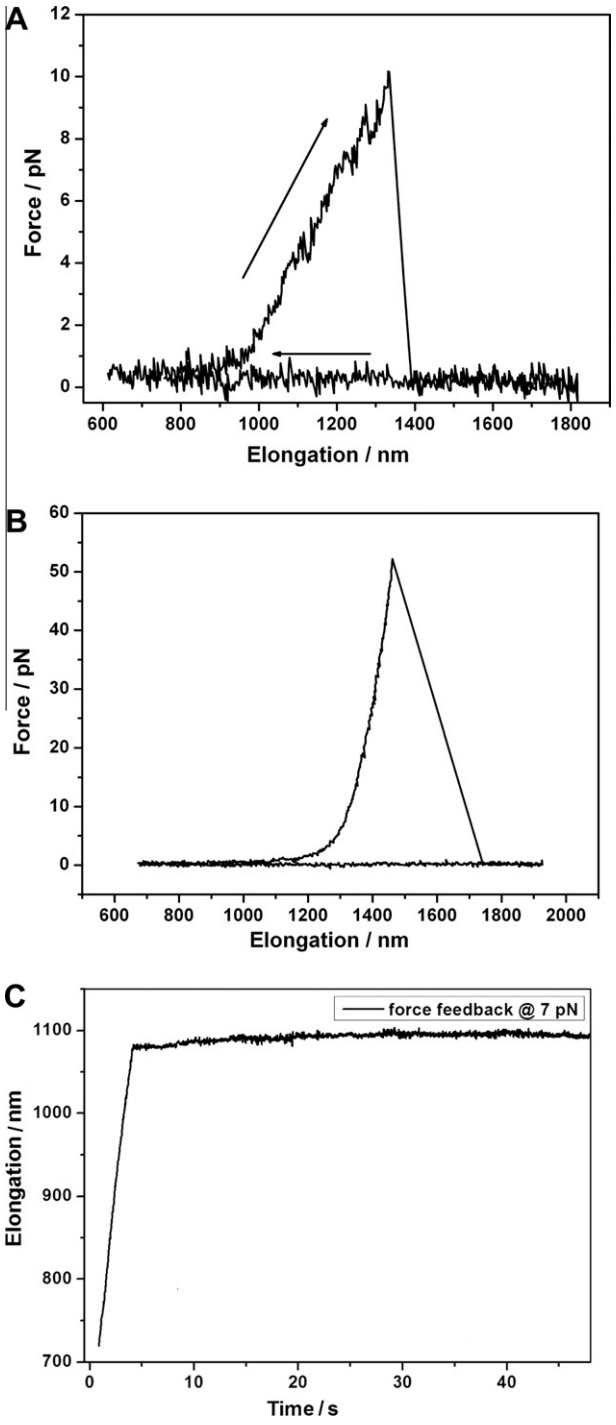

Fig. 2. Disruption of the nascent polypeptide chain from the ribosome and force measurements. (A) A characteristic diagram of force vs. elongation for a specific interaction between the N-terminus of the polypeptide chain and the DNA handle. Coupling ruptures at 10 pN. Arrow pointing upwards indicates extension of the elastic dsDNA linker, whereas the arrow pointing to the left indicates the return of the pipette bead back to its origin. (B) A characteristic diagram of force vs. elongation for a high force rupture of the streptavidin-dsDNA handle from the nascent polypeptide on the ribosome. A distribution between 10 and 50 pN was observed. (C) A diagram of elongation vs. time when a constant force of ~7.0 pN is applied. No rupture is observed through a time of more than 40 seconds which should enable future polypeptide unfolding–refolding experiments while protein is translated. (INSET) Corresponding force versus elongation of the force feedback experiment verifying the elastic nature of the dsDNA handle which is used to couple the protruding polypeptide to the force measuring sphere.

mRNA and the peptidyl-tRNA ruptured, the whole polypeptide chain was uprooted from the ribosomal tunnel.

In order to avoid the rupture in the coupling, we applied a constant force of 7 pN and we monitored the elongation versus time. It became clear that the N-terminus of the polypeptide chain remained coupled to the DNA handle through time (Fig. 2C). By continuing the synthesis it would be possible to measure the forces applied on the nascent polypeptide chain during its elongation, providing that the resolution of the optical tweezers is good enough.

In this study we described a method to immobilize fully active ribosomes on polystyrene beads and hook the N-terminus of a nascent polypeptide chain with a DNA handle attached on a second polystyrene bead trapped by optical tweezers. The described system provides the possibility to measure the forces applied on the nascent chain during its synthesis and potentially during its folding.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.04.045.

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