VIP Protein Folding

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Fast Biosynthesis of GFP Molecules: A Single-Molecule Fluorescence Study**

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Numerous studies have shown that protein folding and maturation can differ substantially between de novo synthesized proteins and in vitro refolded proteins.[1–3] In classical folding studies, formerly folded proteins need to be transferred into an unfolded state before the folding (or rather, refolding) process can be studied. It has been demonstrated in several cases that protein folding already takes place during the elongation of the nascent chain (cotranslational folding). Proteins can become fully folded and enzymatically active while they are still bound to the ribosome through a C-terminal extension of about 30 amino acids that spans the ribosomal channel.[4–7] Significant differences have been observed between folding of de novo synthesized proteins and in vitro refolding with respect to folding rates, the appearance of folding intermediates, and yields.[2,8,9] Therefore, one major goal is to understand how polypeptide chain elongation and folding are coupled. In particular, single-molecule studies can yield valuable information about these rather asynchronous processes. The ribosomal complex as a machine converting the information of the genetic code into a polypeptide chain has already been studied with various single-molecule techniques.[10–14]

Herein, we observed green fluorescent proteins (GFPs) at a single-molecule level after de novo synthesis and folding. Formation of the fluorescent chromophore is a rather slow post-translational autocatalytic process, and the maturation kinetics as well as the folding efficiency differ significantly between GFP wild type and several mutants.[15] We have chosen the GFP Emerald (GFPem) mutant, which is characterized by a high folding efficiency and by fast folding and maturation kinetics.[16,17] GFP synthesis at surface-immobilized fluorescently labeled ribosomes was accomplished using a fractionated cell-free transcription–translation *E. coli* system (Figure 1). The sequence of GFPem was elongated by a sequence of 31 amino acids at the C terminus (spanning the full tunnel length) to ensure proper folding of the full-length protein outside the tunnel.[18] Suppression of protein release after synthesis keeps the synthesized GFP bound to the ribosome and allows us to image GFP fluorescence for extended observation times. For imaging fluorescently labeled ribosomes (with the dye Atto 655) and emerging GFP molecules, we employed a dual-color fluorescence wide-field microscope (Figure 2).[19] Overlaying the ribosome Atto 655 and GFP emission images (Figure 2 d) demonstrates that single GFP molecules are observed colocalized with their

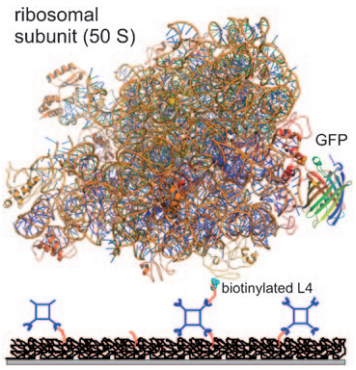

Figure 1. Schematic view of surface-tethered ribosomes (only the 50 S subunit is shown, PDB code: 2AW4). The amino-functionalized cover slide is coated with a layer of poly(ethylene glycol) (PEG) that is biotinylated at low concentration.[27] By the use of a streptavidin–biotin binding assay, fluorescently labeled ribosomes were linked to the surface through biotinylated ribosomal protein L4 (displayed molecules are not to scale). The C-terminal extension of 31 amino acids of our GFP mutant and the suppression of post-translational protein release provides the possibility to observe cell-free-synthesized GFPem, which becomes mature while linked to the ribosome (see the Supporting Information for details).

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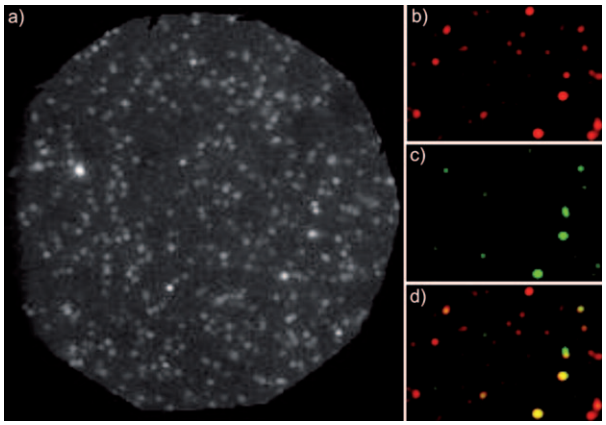

Figure 2. Fluorescence wide-field images from single surface-tethered ribosomes. a) The full screen of the red emission channel showing Atto 655-labeled ribosomes (laser excitation at 640 nm with an exposure time of two seconds). Photobleaching measurements demonstrated that the majority of individual peaks are related to single ribosomes. b) The red emission of ribosomes of a small selected area of image (a). c) For the same area, GFP fluorescence emission is shown, which was measured in the green channel after the transcription–translation reaction had been running for 40 min at 25 °C (laser excitation at 488 nm for 2 s). In both section images (b, c), background was filtered with a band-pass fast Fourier transform (FFT) filter. d) The overlay of the red (ribosomes) and the green (GFP) channel demonstrates that single surface-tethered ribosomes synthesized GFP molecules which become mature (i.e. fluorescent) while bound to the ribosome. The yellow peaks localize the coexistence of single ribosomes and single GFP molecules bound to their synthesizing ribosomes.

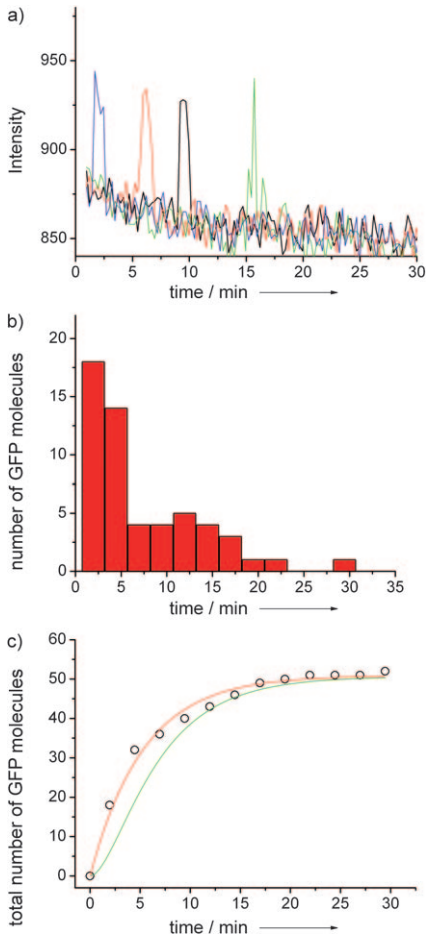

Figure 3. a) As an example, four integrated peak intensities (area of 2×2 pixels) are shown as a function of time for fluorescent GFP molecules appearing at different times after the initiation of biosynthesis. Fluorescence of individual GFP molecules can only be detected for a few consecutive exposures before photobleaching occurs. b) Histogram showing the number of de novo synthesized GFP molecules that appear in consecutive time intervals. After a dead time of about 40 s, a series of exposures was taken every 15 s at room temperature. The resulting appearance times of individual fluorescent GFP molecules, shown in chart (a), were binned into 2.5 min time slices. The shown data originate from five independent biosynthesis experiments. c) The time course of the total number of GFP molecules (○) is fitted by a superposition of two exponential functions describing an irreversible consecutive two-step process:[28]

$$N(t) = \frac{k_2 N_a}{(k_1 - k_2)} \exp(-k_1 t) + \left(\frac{-k_2 N_a}{(k_1 - k_2)} - N_a \right) \exp(-k_2 t) + N_a$$

where N_a is the number of all de novo synthesized GFP molecules and k_1 and k_2 are the rates of the first and the second process. Fitting this function to the experimental data points yields 0.1 min for $1/k_1$ and 5.2 min for $1/k_2$ (fitting curve not shown). This result is rather similar to what we obtained from fits with a single exponential $N(t) = N_a (1 - \exp(-k_1 t))$, which yields 5.3 min for $1/k_1$ (red line). Apparently, the appearance of fluorescent GFP molecules is determined by a single rate constant, which is most probably related to the chromophore formation. Protein synthesis and protein folding must be rather fast (probably faster than one minute), as in a consecutive two-step process, a longer first process for protein folding and subsequent chromophore formation shows a distinct deviation from the experimental data points (see green line, which was calculated with fixed values for $1/k_1 = 2.0$ min and $1/k_2 = 5.3$ min).

synthesizing ribosomes. Our images indicate that approximately 10–15% of all visible ribosomes produce a bound mature and fluorescent GFP. Proper ribosome–GFP complexes remained stable for hours.

In a next series of measurements, we monitored the appearance of individual synthesized GFP molecules as a function of time (Figure 3a). For this purpose, surface-immobilized ribosomes were incubated with a reaction buffer within a closed imaging chamber, and after a dead time of 40 seconds a sequence of images was taken every 15 seconds. The distribution of the appearance time for all detected GFPem molecules is shown in Figure 3b. To our surprise, GFPem fluorescence shows up rather quickly, with a significant fraction within five minutes after initiating polypeptide synthesis. According to the rather limited photostability of GFPem,[16] we observe in most cases photobleaching after a few exposures (Figure 3a) and in some cases also photoblinking. The time course of emerging fluorescent GFPem molecules is satisfactorily fitted by a single exponential (red line in Figure 3c). The corresponding characteristic time constant for the observed process is 5.3 min, which is one of the fastest maturation times for a GFP mutant observed to date. Typical maturation times of other GFP mutants of the S65T type range from 15 to 45 min, whereas wild-type GFP shows even longer maturation times on the order of 2 h.[14,15,20] Fast chromophore formation for GFP is important for its use in kinetics experiments in cells.[21,22]

An interpretation of our result has to consider at least three consecutive subprocesses, namely polypeptide synthesis, protein folding, and chromophore formation, as part of the whole biosynthesis.[*] However, in our study GFP molecules become detectable only after chromophore formation. Therefore, a characteristic time obtained from the kinetic data is related to the succession of all subprocesses. 1) First, we have to account for the polypeptide elongation with a synthesis rate of about one to five amino acid residues per second in cell-free systems, while the corresponding in vivo rate is 10–20 residues per second.[1,22] For our GFP construct with 306 residues (36aa + GFPem + 31aa), synthesis would last one to five minutes for a cell–free system. The synthesis time should not deviate much between different GFP molecules. The time for each step of adding a certain amino acid to the nascent chain varies, but summing up these times for 306 amino acids leads to averaging, and a narrow distribution in the total synthesis time is obtained. The time required for synthesis will appear as a lag time if the time resolution of the measurement is sufficiently high. In our measurements, we observed first mature GFP molecules after one minute. Owing to a limited time resolution (ca. 40 seconds dead time), we do not observe such a lag time (Figure 3c). Thus, we conclude that polypeptide synthesis in our assay proceeds in a time not longer than one minute. This synthesis rate is close to that observed under in vivo condition. 2) Folding rates of GFP are typically known from refolding studies. The corresponding times range from four to five minutes for concentrated proteins in solution[23,24] to a few tens of seconds in single-molecule studies[25] or in chaperonin-mediated refolding.[26] As demonstrated in Figure 3c, protein folding seems to be fast in our approach, similar to results observed earlier in folding studies on single GFP molecules.[25] 3) As the characteristic chromophore formation requires at least 5–10 min for de novo synthesized GFP molecules,[24] we have to assume that the characteristic time constant obtained from our data (5.3 min) is related to the chromophore formation. To analyze the impact of processes preceding chromophore formation (i.e. protein synthesis and folding) on the time course of the whole biosynthesis process, we fitted a function describing an irreversible, consecutive, two-step process to the experimental data. In this model, the first process is related to protein synthesis and protein folding, and the second process represents chromophore formation. The results of fitting this model to our data yield a short characteristic time ($1/k_1 =$ 0.1 min) and a long characteristic time ($1/k_2 = 5.2$ min), which is in agreement with the fit using a single exponential. Therefore, we conclude that polypeptide synthesis and protein folding together must be faster than one minute. Assuming longer characteristic time constants for protein synthesis or folding (e.g., $1/k_1 = 2$ min) leads to a distinct deviation from the experimental data (green line in Figure 3c). Our results demonstrate that cotranslational protein folding and maturation is characterized by fast kinetics. Conventional ensemble refolding studies with proteins at moderate concentrations usually exhibit slower folding and maturation kinetics.[24] High rates of folding and maturation are assumed to play a crucial role in reducing unwanted side reactions, such as misfolding and aggregation, and thereby improve the efficiency of protein biosynthesis in the cell.

Two-color wide-field imaging of functional surface-immobilized ribosomes allowed us to measure fluorescence of single molecules for extended time periods, thus monitoring biosynthesis of GFP, which becomes fluorescent only after a final autocatalytic process of the chromophore formation. A remarkably short characteristic maturation time of about 5 min is observed for the GFPem mutant. If the time course of cellular events is fast, it is of vital importance to employ GFP molecules with maturation times as fast as possible. Even, if as in our study, the characteristic time constant is about 5 min, we can follow the time courses of those GFPs which already show fluorescence within one minute.

[*] Transcription takes place in the reaction buffer before the sample is injected into the imaging chamber in which the biosynthesis is started. Therefore, we do not have to consider the time course of this process.

Experimental Section

Ribosomes from *E. coli* were biotinylated at the L4 protein, labeled with Atto655 (Atto Tec, Siegen, Germany), and tethered to a PEG-covered glass slide through a streptavidin–biotin binding assay. Using a modified plasmid pRSET/EmGFP (Invitrogen) and a transcription–translation fractionated system from RiNA GmbH (Berlin, Germany), GFP Emerald including a C-terminal extension of 31 amino acids was synthesized by surface-tethered ribosomes (see the Supporting Information for details).

For single-molecule imaging, we employed excitation light from an Ar^+ ion laser at 488 nm and at 640 nm from a dye laser (pumped by the argon laser). Using an inverted microscope (IX-81, Olympus, Germany) in wide-field illumination mode and a custom-made dichroic wedged mirror (Omega Optical, Brattleboro, VT, USA), we imaged fluorescence emission of single surface-tethered ribosomes and of associated GFP molecules by projecting two separate images (red and green emission channels) on a back-illuminated CCD camera (Figure S1 in the Supporting Information).[19]

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