Single-Molecule Studies

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Light at the End of the Tunnel

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Protein folding, that is, how a linear sequence of amino acids is transformed into a protein with a three-dimensional structure that has a specific function, is an exciting research area, and has been the subject of intensive studies.[1,2] Most of the present knowledge on protein folding is derived from refolding studies of proteins that have been denatured from their native folded state. As the number of configurations accessible for a denatured protein is very large, the structure of a protein refolded after denaturation is not fully comparable with a that of a peptide chain that folds after biosynthesis by the ribosome. The difference lies in the very important aspect of postbiosynthetic folding that is termed cotranslational folding: the first folding steps occur after only a short sequence of the entire polypeptide chain has been synthesized.[3] Cotranslational folding influences which reaction coordinate the polypeptide sequence chooses for its initial folding steps and also affects the speed of the whole folding process while the ribosome continues to translate the messenger RNA.

Folding of de novo synthesized proteins is best studied in a natural environment, ideally in a living cell. An intermediate approach is studying translation in vitro, which can be realized by organizing isolated ribosomes on glass surfaces or in artificial membranes and operating them under cell-free conditions.[4] In this way, protein biosynthesis and folding studies are simplified, and the system can be readily manipulated and modified. Typically, peptide biosynthesis by ribosomes in a cell-free environment is slowed down to about 1 to 5 amino acids per second, compared to 10 to 20 amino acids per second in a living cell.[3]

Substantial knowledge in protein folding has been gained in the recent past from single-molecule experiments. Single-molecule techniques, ranging from atomic force microscopy (AFM) to single-molecule fluorescence techniques, make it possible to study biomolecules without ensemble averaging or required synchronization. In particular, single-molecule fluorescence methods have become unique tools to study biomolecular interactions and conformational changes at the level of a single system with a minimum of invasion and with high temporal and spatial resolution.[5–7]

A large spectrum of single-molecule methods have been applied to investigate the folding of individual proteins. Out of the toolbox of single-molecule fluorescence methods, fluorescence resonance energy transfer (FRET) and photo-induced electron transfer (PET) are ideal tools to probe conformational changes at the nanometer scale as well as fast folding kinetics,[7] and have been used intensively for protein folding studies.[8–10] However, previous single-molecule fluorescence studies on protein folding required the chemical introduction of a fluorescent label into a protein or a polypeptide chain. Not only might the label interfere with unfolding and refolding processes, but it also cannot be used to study folding immediately following biosynthesis. As an alternative single-molecule method that does not require labeling with a fluorescent probe, atomic force microscopy has been used to study unfolding pathways of individual protein molecules.[11]

The folding mechanism and kinetics of green fluorescent protein (GFP) and its colorful derivatives are of particular interest, as this large class of fluorescent labels is widely used in many areas of biological research.[12,13] Some insights into the unfolding pathway of single GFP molecules have been gained using AFM combined with protein engineering in order to "lock" certain structural submotifs.[14] To become fluorescent, following biosynthesis and folding, GFP and other fluorescent proteins must undergo an autocatalytic maturation that generates the chromophore from the condensation of usually three amino acid residues. A remaining concern here is the relatively long maturation time of fluorescent proteins, which limits their use for live-cell microscopy of faster processes and makes the design and characterization of fast-maturing fluorescent proteins very desirable.

A very elegant strategy to study the folding and maturation of GFP right after biosynthesis has now been reported by Katranidis et al.[15] They designed an experiment to observe the whole process of translation at the single-molecule level, including the synthesis of a polypeptide chain, cotranslational folding, and maturation of the protein to its full functionality, using fluorescence microscopy. In this experiment, they attached single ribosomes, which were chemically labeled with a red fluorophore, to a glass surface and surrounded by a reservoir that contained a cell-free transcription–translation system (Figure 1). The fully folded and matured GFP

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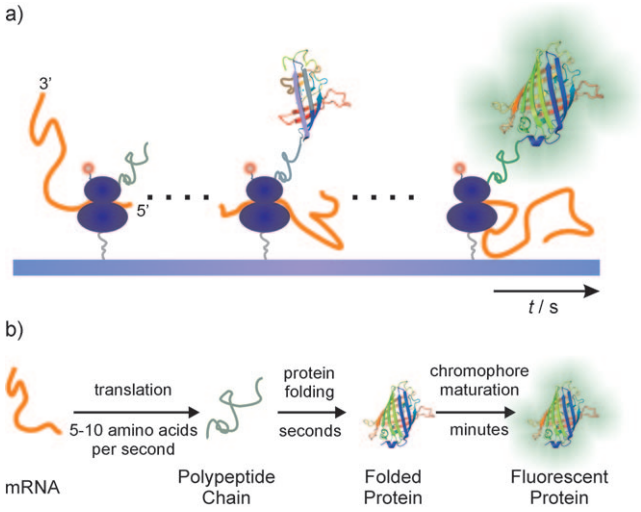

Figure 1. a) Representation of the assay that allows observing the synthesis and maturation of a single GFP Emerald protein. Single, fluorophore-labeled ribosomes were attached to a glass surface in a reservoir containing a cell-free transcription–translation system, together with a plasmid that encodes for the protein. The transcribed mRNA is processed by the ribosomes, and a polypeptide chain is synthesized. Shortly after the beginning of polypeptide synthesis, cotranslational folding of the nascent polypeptide chain starts. After the protein is fully folded, an autocatalytic maturation leads to the formation of the chromophoric unit of GFP Emerald, turning the protein fluorescent. b) Kinetic data of the maturation time of GFP Emerald was derived from temporal fluorescence microscopy studies. Single-exponential kinetics with a characteristic time constant of 5.3 min was determined. The decay time is attributed to the rate-limiting step of chromophore maturation.

molecule could then be detected by its own characteristic fluorescence, without the need for any labeling reaction. Furthermore, the researchers chose GFP Emerald, a fast-folding and -maturing variant of GFP. A crucial point in this strategy is keeping the synthesized and folded protein at the ribosome to ensure sufficient time for the protein to fold and mature into its final state, and for detection of the final protein through its fluorescence signal. This has been achieved by extending the plasmid of GFP Emerald with a 31 amino acid sequence, which locks the polypeptide chain after synthesis in the ribosomal channel. In this way the researchers were able to observe the synthesis of fully functional single GFP Emerald proteins that appeared as fluorescence signals colocalized with the fluorescently labeled ribosomes. Overall, more than 10% of all ribosomes at the glass surface synthesized an intact GFP molecule.

In a further experiment, Katranidis et al. studied the kinetics of the appearance of single fluorescent GFP Emerald proteins by observing the fluorescence signal with respect to the start of translation over time. They determined single-exponential kinetics with a characteristic time constant of 5.3 minutes for the whole process of translation, before the fluorescence signal of GFP Emerald appeared. In addition, they observed a significant fraction of proteins that were already fully matured after only one minute. From this kinetic data, the researchers reconstructed the steps of protein biosynthesis in detail and came to interesting new conclusions: 1) the in vitro peptide synthesis occurs with surprisingly high speed and in less than one minute, which is close to what is observed in vivo; 2) protein folding of GFP Emerald is very fast and supported by cotranslational folding, such that the characteristic time of 5.3 minutes mainly corresponds to the maturation of the chromophore. In conclusion, the GFP Emerald mutant is characterized by very fast maturation kinetics, which makes this protein very attractive for studying relatively fast cellular events that are typically inaccessible owing to the slow maturation time of most other GFP mutants.

The impact of this work is manifold. The experimental strategy to observe individual fluorescent proteins fold is the prerequisite for future work on engineering and characterizing novel mutants of fluorescent proteins. Novel mutants that exhibit fast folding and maturation kinetics are in particular desirable for the observation of faster processes in vivo. Moreover, it is possible to analyze the folding pathway of fluorescent proteins in more detail, and importantly, right after biosynthesis by a ribosome. Extended studies might pave the way towards a refined understanding of the folding and maturation mechanisms of fluorescent proteins.

One could imagine generalizing the approach for folding studies of other proteins that do not exhibit fluorescence, for example, by using fluorescent amino acid derivatives or by introducing fluorescent reporters postbiosynthetically. The use of multiple reporters with different spectral properties might enable the observation of folding intermediates of the nascent and growing polypeptide chain by probing small distance changes using FRET and multi-FRET.[16] Alternatively, fluorescently labeled tRNAs could be used to observe and possibly correlate peptide-chain elongation and the first steps of cotranslational protein folding. A combination with high-resolution fluorescence microscopy, for example, methods that have demonstrated to localize a molecule with an accuracy of about one nanometer[17] or to allow imaging below the diffraction limit,[18,19] might provide a real-time and high-resolution microscopic picture of the first moments of a nascent protein.

In conclusion, the paper by Katranidis et al. demonstrates that there is ample scope for further experiments, and we surely can expect many new applications and a more fundamental insight into polypeptide-chain biosynthesis, protein folding and maturation in the future.

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[1] K. A. Dill, S. B. Ozkan, M. S. Shell, T. R. Weikl, *Annu. Rev. Biophys.* **2008**, *37*, 289.
[2] A. R. Fersht, V. Daggett, *Cell* **2002**, *108*, 573.
[3] A. N. Fedorov, T. O. Baldwin, *J. Biol. Chem.* **1997**, *272*, 32715.
[4] R. A. Marshall, C. E. Aitken, M. Dorywalska, J. D. Puglisi, *Annu. Rev. Biochem.* **2008**, *77*, 177.
[5] N. G. Walter, C. Y. Huang, A. J. Manzo, M. A. Sobhy, *Nat. Methods* **2008**, *5*, 475.
[6] S. Weiss, *Science* **1999**, *283*, 1676.
[7] P. Tinnefeld, M. Sauer, *Angew. Chem.* **2005**, *117*, 2698; *Angew. Chem. Int. Ed.* **2005**, *44*, 2642.
[8] X. Michalet, S. Weiss, M. Jager, *Chem. Rev.* **2006**, *106*, 1785.
[9] B. Schuler, W. A. Eaton, *Curr. Opin. Struct. Biol.* **2008**, *18*, 16.

[10] G. Ziv, G. Haran, *J. Am. Chem. Soc.* **2009**, *131*, 2942.
[11] F. Oesterhelt, D. Oesterhelt, M. Pfeiffer, A. Engel, H. E. Gaub, D. J. Müller, *Science* **2000**, *288*, 143.
[12] R. Tsien, *J. Cell Biol.* **2007**, *179*, 6.
[13] G. U. Nienhaus, *Angew. Chem.* **2008**, *120*, 9130; *Angew. Chem. Int. Ed.* **2008**, *47*, 8992.
[14] M. Mickler, R. I. Dima, H. Dietz, C. Hyeon, D. Thirumalai, M. Rief, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 20268.
[15] A. Katranidis, D. Atta, R. Schlesinger, K. H. Nierhaus, T. Choli-Papadopoulou, I. Gregor, M. Gerrits, G. Buldt, J. Fitter, *Angew. Chem.* **2009**, *121*, 1790; *Angew. Chem. Int. Ed.* **2009**, *48*, 1758.
[16] M. Heilemann, P. Tinnefeld, G. Sanchez Mosteiro, M. Garcia Parajo, N. F. Van Hulst, M. Sauer, *J. Am. Chem. Soc.* **2004**, *126*, 6514.
[17] A. Yildiz, P. R. Selvin, *Acc. Chem. Res.* **2005**, *38*, 574.
[18] M. Heilemann, P. Dedecker, J. Hofkens, M. Sauer, *Laser Photonics Rev.* **2009**, *3*, 180.
[19] S. W. Hell, *Nat. Methods* **2009**, *6*, 24.

