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Unraveling co-translational protein folding: Concepts and methods

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ABSTRACT

Advances in techniques such as nuclear magnetic resonance spectroscopy, cryo-electron microscopy, and single-molecule and time-resolved fluorescent approaches are transforming our ability to study co-translational protein folding both *in vivo* in living cells and *in vitro* in reconstituted cell-free translation systems. These approaches provide comprehensive information on the spatial organization and dynamics of nascent polypeptide chains and the kinetics of co-translational protein folding. This information has led to an improved understanding of the process of protein folding in living cells and should allow remaining key questions in the field, such as what structures are formed within nascent chains during protein synthesis and when, to be answered. Ultimately, studies using these techniques will facilitate development of a unified concept of protein folding, a process that is essential for proper cell function and organism viability. This review describes current methods for analysis of co-translational protein folding with an emphasis on some of the recently developed techniques that allow monitoring of co-translational protein folding in real-time.

Contents

Abbreviations: (aa)-tRNAs, aminoacyl-tRNAs; AFM, atomic force microscopy; CFP, cyan fluorescent protein; cryo-EM, cryo-electron microscopy; FRET, Fluorescence Resonance Energy Transfer; mRNA, messenger RNA; NMR, Nuclear Magnetic Resonance spectroscopy; RNC, ribosome-bound nascent chain complex; SMFS, single-molecule force spectroscopy; YFP, yellow fluorescent protein.

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1. Introduction

Proteins are polymers of amino acids covalently linked by amide bonds. Most proteins are compactly folded, with specific secondary and tertiary structures that are essential for the protein's function. A correctly folded proteome largely defines the functionality of a cell and the phenotype of an organism. Misfolding of proteins contributes to the development of numerous diseases including neurodegenerative diseases, cancer, and type 2 diabetes mellitus [1,2]. While our knowledge of how proteins acquire their final structure remains incomplete, substantial progress has been made both in understanding the process of protein folding and prediction of protein structures [3–7]. This information has resulted primarily from *in vitro* denaturation/renaturation [8] and computer-based simulation experiments [6,7], which were historically the main approaches used in the field [3–8]. However, a comprehensive understanding of protein folding requires elucidation of the folding mechanism under native intracellular conditions, where protein folding is influenced by many factors and multifactorial processes [5,9,10].

In vivo protein folding differs significantly in a number of its basic characteristic features from the refolding process in a test tube [9,10]. Most importantly, *in vivo* protein folding is widely believed to start during protein synthesis on the ribosome, i.e., co-translationally [11–18]. Co-translational folding is thus tightly coupled to the dynamics of protein synthesis and therefore is believed to be affected by kinetics of translation elongation [12,13,16–21]. *In vivo* protein folding is a vectorial process; i.e. the polypeptide chain is synthesized and is believed to be folded predominantly from the N-terminal to the C-terminal end [11–18]. Co-translational folding of a nascent polypeptide thus results in sequential structuring of distinct regions of the polypeptide emerging from the ribosome at different points in time [11–18]. Importantly, co-translational protein folding begins very early during the process of polypeptide chain synthesis on the ribosome, with some secondary structure elements (e.g., alpha-helices) forming inside the ribosomal tunnel and some tertiary structures forming as early as in the vestibule region of the tunnel, and thus in many cases it is believed to follow the framework (hierarchic) model [11–18]. Finally, the ribosomes, folding catalysts, and molecular chaperones might interact with the synthesized chains and affect their folding [9–11,22–24]. Therefore, studies of *in vivo* co-translational protein folding are much more challenging than *in vitro* refolding studies not only because of the vectorial nature of *in vivo* co-translational folding, but also because it takes place in a crowded cellular environment. Thus, in addition to other parameters affecting co-translational folding, excluded volume effects have a substantial impact on the folding mechanism [9–11].

In the early 1960s and 1970s, the first observations were made suggesting that *in vivo* protein folding, at least for some proteins, is a co-translational process [25–31]. The majority of these early experiments involved isolation/fractionation of ribosome-bound nascent chain complexes (RNCs) through a sucrose density gradient, followed by assessment of the structural properties of the nascent chains through measurement of i) their specific enzymatic activities [25–27], ii) their recognition by specific/conformational antibodies [28], or iii) formation of correct disulfide cross-bridges within and/or between nascent chains [29–31]. Subsequently, other methods have been introduced involving e.g., measurement of (i) the resistance of RNCs to proteolytic digestion [32–34]; (ii) the ability of co-factors and ligands (such as heme) to bind the growing polypeptide chain (as an indication that a binding-competent conformation has been achieved) [35,36], and/or (iii) the ability of nascent chains to form oligomeric complexes with other polypeptides (as an indication that the surfaces/shapes responsible for intersubunit interactions/contacts have been formed) [37–39]. More recently, NMR spectroscopy [40–42 and Ref. therein], cryo-electron microscopy (cryo-EM) [43–45 and Ref. therein], fluorescent techniques (e.g., Fluorescence Resonance Energy Transfer (FRET) [46–49 and Ref. therein]), and fluorescence anisotropy/dynamic fluorescence depolarization [50–52 and Ref. therein], as well as some other approaches (see below) have been used to assess the conformation and dynamics of polypeptides emerging from the ribosome during translation. These approaches provided overwhelming data in support of co-translational folding. It should be noted, however, that most of these studies involved "steady-state" experiments and used RNCs isolated through affinity chromatography and/or a sucrose density gradient centrifugation requiring a substantial amount of time (typically several hours). Thus, although the information obtained using these methods was extremely useful for understanding the dynamics of nascent chain folding, it could not be excluded that, in certain cases, nascent chains acquired their specific structural features during RNC isolation and not during the process of translation *per se*. This highlights the importance of developing and applying new *in situ* real-time approaches to answer remaining key questions related to co-translational folding (e.g., what structures are formed during protein synthesis and when are they formed?). Here, I briefly review the techniques currently available to study co-translational folding, with an emphasis on some of the recently devised methods that allow monitoring of protein folding in real-time.

2. Overall strategy for studying co-translational protein folding

Pioneering experiments performed by Cowie et al. [25], Zipser and Perrin [26], and Kiho and Rich [27] in the early 1960s established a basic set of requirements for methods aimed at studying co-translational folding; this set of requirements has remained largely unchanged to date. First, there should be an easily measurable means for assessment of proper folding of nascent chains on the ribosome (e.g., acquisition of enzymatic activity and/or appearance of specific structural epitopes). Second, it must be ensured that the specific structural features under investigation are indeed attributable to the ribosome-bound nascent chains and not to polypeptide chains bound to ribosomes/polyribosomes nonspecifically. Thus, it must be verified that the protein under investigation is not simply associated (e.g., co-sedimenting) with ribosomes, but is a product of active synthesis on ribosomes. This is typically addressed by testing whether protein detachment from the ribosome (e.g., using the antibiotic puromycin which causes chain termination and release) leads to release of the ribosome-bound protein specific "structural feature(s)/activity". Third, the polypeptide chains should be synthesized *de novo* to ensure that outcomes being measured are truly the result of a co-translational process. In order to ensure that measurements of co-translational folding are performed with ribosome-bound nascent chains, an additional set of tools was developed. These included mRNAs lacking a stop codon

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