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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

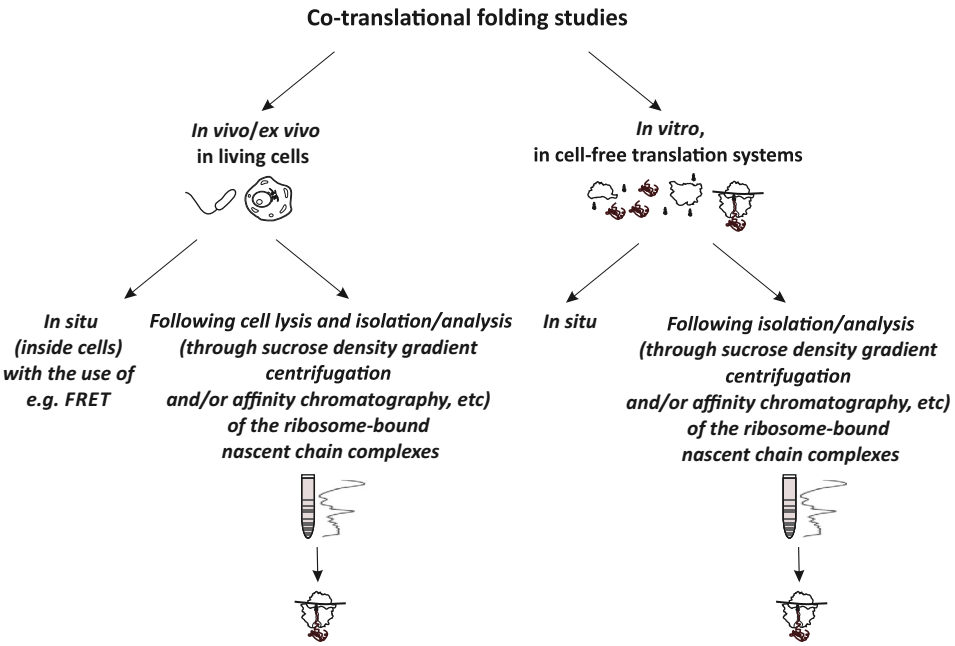


Fig. 1. General strategies for studying co-translational protein folding. Experiments can be performed *in vivo* (in living cells) or *in vitro* (in cell-free systems) and involve *in situ* measurements or measurements following isolation of ribosome-bound nascent chain complexes.

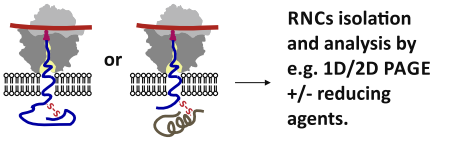
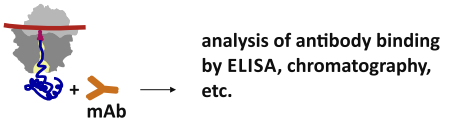
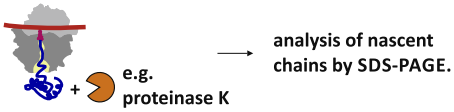
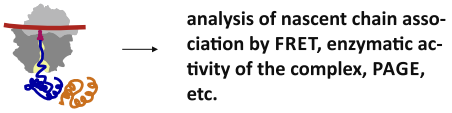
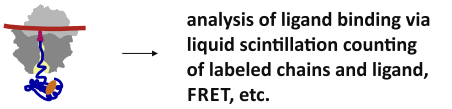
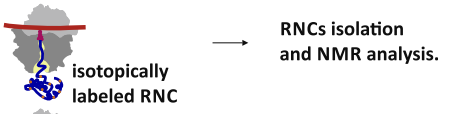
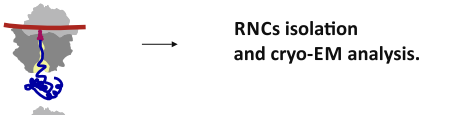
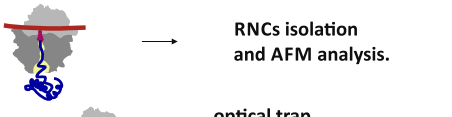
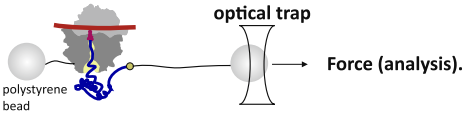
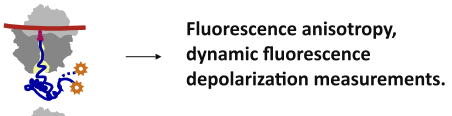
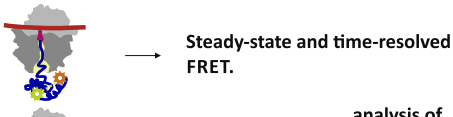
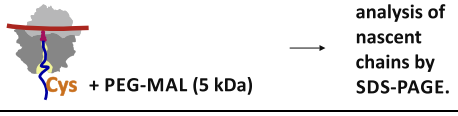
(non-stop mRNAs) and/or containing so-called ribosome-stalling or "arrest sequences" to prevent release of nascent chains from the ribosome [for a review see 53–55]. Without a termination codon, protein synthesis stops but the completed protein is not released from the ribosome (for several hours, at least). One has to take into account, however, that all organisms have evolved various mechanisms to recognize stalled ribosomal complexes and initiate pathways leading to eventual chain release and ribosome recycling [56–58]. Thus, it has to be carefully ensured that measurements are done with true RNCs. The so-called ribosome-stalling sequences can halt translation elongation when inserted into (or fused to the C-terminus) of virtually any protein [53–55]. Bacterial SecM, TnaC, RrmC and MifM and eukaryotic (*Neurospora crassa*, *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *Cryptococcus neoformans*) AAPs (Arg-attenuator peptides) are the best known examples of such regulatory sequences [53,55,59,60 and Ref. therein]. The use of non-stop mRNAs and ribosome-stalling sequences (especially SecM) was instrumental for isolation of RNCs carrying nascent chains of predetermined sizes for their subsequent analysis using e.g., NMR [42] and other approaches.

2.1. In vivo versus in vitro measurements

The basic strategies described above have been applied for decades to study co-translational protein folding both in cells and *in vitro* in reconstituted cell-free translation systems (Fig. 1). Early experiments typically used cellular systems (e.g., *Escherichia coli* and/or mammalian cells) and involved induction of synthesis of a specific protein (or radioactive pulse-labeling of nascent chains in the context of constitutive protein synthesis) followed by monitoring of protein expression and analysis of the polysomal distribution of nascent chains and the specific activity and/or specific structural properties of the chains [25–31]. In a seminal experiment published by Kiho and Rich [27] in 1964 (which utilized *E. coli* cells), polysomal-bound β-galactosidase activity was found in a sharply defined "heavy" polysomal peak region of the sucrose gradient following induction of β-galactosidase synthesis [27]. No β-galactosidase enzymatic activity was found in this polysomal region without induction of β-galactosidase synthesis. These results suggested that the observed enzymatic activity was confined to newly synthesized β-galactosidase protein that acquired its activity during protein synthesis on the ribosomes. At the time of this study, it was known that active β-galactosidase is comprised of 4 subunits. Therefore, the authors proposed a mechanism involving interaction between the nascent chains on the same (or different) polysomes, in the course of which completed monomer units (after translation termination) were transferred from one ribosome to an adjoining and/or nearby ribosome to form the active tetramer [27]. The authors concluded "that an additional stage of protein synthesis is occurring on the polysome in which there is not only the assembly of a single polypeptide chain but also a secondary assembly of these monomers to form an enzymatically active molecule" [27]. While this experiment provided one of the first observations supporting co-translational folding, it involved a ~2.5 h sucrose density gradient centrifugation step [27] and thus was not truly *in vivo* or *in situ*. Under these circumstances, it was not possible to exclude the possibility that active β-galactosidase was formed during the RNC isolation step and not concomitantly with protein synthesis.

While admitting that the time required for cell lysis and nascent peptide isolation was a major deficiency in several previous experiments, researchers continued to utilize cellular experimental setups in the next decade. Of note, robust cell-free expression systems such as the *E. coli* system reported by Zubay in 1973 [61] and the Rabbit Reticulocyte Lysate (RRL) system developed by Pelham and Jackson in 1976 [62]) were either not yet developed or in their infancy at this point in time. In the mid 1970s, Bergman and Kuehl pioneered experiments using eukaryotic cellular systems rather than the earlier prokaryotic systems [29–31]. These experiments were aimed at assessment of disulfide bonds formation in nascent secretory proteins in eukaryotic cells, specifically in immunoglobulin polypeptides expressed in mouse myeloma cells [29–31]. Using a combination of fast centrifugation and chromatography techniques to isolate membrane-bound ribosomes/nascent polypeptides, they showed that formation of correct inter- [29] and intra-chain disulfide bonds [30,31] within immunoglobulin

Table 1
Key methods available for measuring co-translational protein folding. Each method is described by a schematic illustration of the experimental set up and comments on its possible applications.

Method	Experimental set-up	Application
Enzymatic activity		Applicable *in vitro* and *in vivo*. Suitable for *in situ*, real-time measurements. Requires extrusion of the substantial portion of the nascent polypeptide chain out of the ribosomal tunnel. Not applicable for investigation of nascent chain conformation inside the tunnel.
Disulfide bond formation		Applicable mostly *in vivo* for detection of intra- and intermolecular disulfide bonds. Requires isolation of RNCs. Not applicable for investigation of nascent chain conformation inside the tunnel.
Detection of structural epitope formation via conformational antibodies		Applicable *in vitro* and *in vivo*. As a rule, requires preparation and isolation of stalled RNCs. Extensive tests of the conformational antibodies are warranted to ensure that they are not inducing formation of the conformational epitopes.
Limited proteolysis		Requires preparation and isolation of stalled RNCs. Applicable for investigation of nascent chain conformation inside and outside the tunnel.
Formation of oligomeric complexes		Applicable *in vitro* and *in vivo*. Suitable for *in situ* measurements. Not applicable for investigation of nascent chain conformation inside the tunnel.
Ligand binding		Applicable *in vitro* and *in vivo*. Suitable for *in situ* measurements. Not applicable for investigation of nascent chain conformation inside the tunnel.
NMR spectroscopy		Applicable for analysis of RNC complexes prepared *in vitro* and *in vivo*. In general, applicable for investigation of nascent chain conformation inside and outside the tunnel.
Cryo-electron microscopy (cryo-EM)		Applicable for analysis of RNC complexes prepared *in vitro* and *in vivo*. In general, applicable for investigation of nascent chain conformation inside and outside the tunnel.
Atomic force microscopy (AFM)		Applicable for analysis of RNC complexes prepared *in vitro* and *in vivo*. In general, applicable for investigation of relatively large nascent chains outside the tunnel.
Single molecule force spectroscopy		Applicable for analysis of RNC complexes prepared *in vitro*. Suitable for investigation of folding of relatively large nascent chains outside the tunnel.
Fluorescence anisotropy/dynamic fluorescence depolarization		Applicable for analysis of RNC complexes prepared *in vitro*. Suitable for investigation of the local dynamics of the ribosome attached nascent proteins.
FRET		Applicable for studies of co-translational folding *in vitro* and *in vivo*. Especially suitable for time-resolved kinetic measurements of co-translational folding. Applicable for investigation of nascent chain conformation inside and outside the tunnel.
Cysteine accessibility assay		Applicable for analysis of RNC complexes prepared *in vitro*. Mostly suitable for investigation of nascent chain conformation inside the tunnel.

light and heavy polypeptide chains occurs rapidly after the involved cysteine residues pass through the membrane into the cisterna of the endoplasmic reticulum.

Many subsequent studies employed *E. coli* and/or eukaryotic (mostly mammalian) cell systems to study co-translational folding, but most were limited in their interpretation by the required cellular lysis and nascent chain purification/isolation steps. True *in situ* experiments that avoid lengthy purification steps and may allow real-time measurements of co-translational folding were eventually made possible by development of a variety of cell-free translation systems (both prokaryotic and eukaryotic) [reviewed in 12–18]. While still having some drawbacks (e.g., *in vitro* protein synthesis reactions often are not as efficient as cellular reactions and RNC purification is still necessary in some experiments), cell-free translation systems offer numerous advantages. First, they allow direction of the protein synthesis machinery to translation

of a single exogenous mRNA of interest. Second, they are amenable to pathway engineering, allowing modulation of the presence and specific concentrations of various components of the cellular machinery (e.g. translation factors, chaperones, etc.). Finally, they allow for incorporation of non-natural amino acids into specific positions of an *in vitro* synthesized protein through the use of engineered aminoacyl-tRNAs. Such tRNAs can be pre-acylated with non-natural amino acids using natural enzymes [18,47,63,64 and Ref. therein] or engineered ribozymes (so called "Flexizymes") [65]. tRNAs incorporating amino acids with fluorescent-labels have been especially instrumental in developing real-time approaches to monitor co-translational protein folding [46–49 and Ref. therein].

3. Experimental methods for detection of co-translational folding of nascent polypeptide chains

Key methods available for measuring co-translational protein folding are summarized in Table 1.

3.1. Enzymatic activity

Acquisition of enzymatic activity by ribosome-bound nascent chain(s) provided the most solid and direct initial support for the phenomenon of co-translational protein folding. Early experiments involving polysome-bound β-galactosidase (described above) [25–27] were followed by many other studies, which assessed the activities of firefly [32,34,66–68] and/or bacterial luciferases [69–71], mammalian rhodanese [72], Ricin A-Chain [73], Semliki Forest virus (SFV) capsid (C) protein [74,75], Green Fluorescent Protein (GFP) [76–78] and some other proteins. Several reports are of special interest. The study by Kolb, Makeyev and Spirin was one of the first attempts to investigate co-translational protein folding in real-time in a cell-free system [66]. The authors developed a technique to continuously monitor enzymatic activity of newly synthesized firefly luciferase in a cell-free system in a luminometer cuvette [66]. Luciferase activity indicative of folding of the protein was detected as soon as the full-length molecule was formed in the translation reaction [66]. Addition of RNase A abrogated both translation and accumulation of active luciferase. While the authors were unable to detect any luciferase activity in the ribosome-bound chains (the last 12 C-terminal amino acids are important for activity of the enzyme [66 and Ref. therein]), they nevertheless found that luciferase became active immediately after the release of the nascent chains [66]. Importantly, such rapid acquisition of the enzyme's activity was incompatible with a post-translational folding scenario, as refolding of the enzyme from the denatured state required more than 10 min [66]. Subsequently, in a separate study, the same research group demonstrated that ribosome-bound luciferase can be enzymatically active, but this requires extension of its C-terminus by at least 26 additional amino acids, allowing extrusion of the C-terminal end (important for luciferase activity) out of the ribosomal tunnel [67]. The authors concluded that folding of the firefly luciferase protein occurs during the course of translation [66,67]. Later, Nicola, Chen and Helenius [74] provided one of the first *in situ* observations of co-translational folding in the cytosol of living cells by employing an "enzymatic activity approach". They took advantage of the fact that Semliki Forest virus capsid protein (C protein) contains a chymotrypsin-like protease domain that must fold before it can auto-catalytically cleave itself from a larger polyprotein precursor [74 and Ref. therein]. The C protein (267 amino acids) is the most amino-terminal of the five SFV proteins comprising the 1257 amino acid-long polyprotein precursor [74 and Ref. therein]. Using a combination of *in vivo* [^{35}S]methionine and [^{35}S]-cysteine pulse-chase labeling of the Semliki Forest virus polyprotein expressed in SFV-infected Chinese hamster ovary (CHO) cells and immunoprecipitation approaches, the authors demonstrated that C-protein cleavage occurs when the growing nascent chain reaches a length of ~310 amino acids [74]. As the ribosome tunnel occludes the ~30–40 most C-terminal residues of a nascent polypeptide [79], this data indicates that that C protein cleaves itself off almost immediately after its C-terminal end (the cleavage site) extrudes out of the ribosome tunnel and led the authors to conclude that the protease domain of the SFV C protein folds co-translationally in the cytosol of living cells [74].

3.2. Disulfide bond formation

Pioneered by Bergman and Kuehl [29–31], analysis of disulfide (S—S) bond formation has been extensively used to study co-translational folding of secretory proteins both in cells and in cell-free systems, making it a paradigm for understanding the protein maturation and quality control processes of the secretory pathway [29–31,80–85]. The formation of correct disulfide bonds in growing nascent chains is an indication that compact structures (characteristic of the folded protein) that allow for close proximity of distant cysteines are formed during protein translation. To obtain information about the extent of disulfide bond formation in nascent chains, several approaches have been used. Almost all start with radioactive pulse labeling of the synthesized chains followed by their isolation by immunoprecipitation or chromatography/centrifugation. The isolated chains are then analyzed for the presence of specific S—S bonds using tryptic digestion [29] and/or 1D/2D gel electrophoresis performed in the presence/absence of a reducing agent (covalent intramolecular disulfide bonds result in more compact structures within the nascent chains leading to faster migration upon non-reducing SDS-PAGE) [30,31,80–85]. Studies using these methods provided direct biochemical evidence for co-translational folding of nascent chains and co-translational disulfide bond formation for a number of proteins, including immunoglobulins (heavy and light chains) [29–31], serum albumin [80], influenza hemagglutinin [81,82,85], low-density lipoprotein receptor [83], HIV-1 envelope glycoprotein [84], and others. Moreover, this approach allowed the order of S—S bond formation to be determined in several proteins and demonstrated that hierarchical S—S bond formation may help prevent nonproductive interactions that might lead to protein misfolding [84,85].

3.3. Detection of structural epitope formation using conformation-specific antibodies

The original idea to use immunological probes to detect specific structures within RNCs that would indicate co-translational folding of nascent chains was developed by Hamlin and Zabin [28], who attempted to check for the presence of specific epitopes within β-galactosidase nascent chains. However, since they used a polyclonal immune serum, it was difficult to ascertain the specificity of the immunological probe for the native protein conformation. Later, Fedorov and Goldberg [86] introduced the use of a conformation-dependent monoclonal antibody (recognizing specific structures with the small ~11.5 kDa N-terminal fragment) to monitor co-translational folding of a 43 kDa tryptophan synthase beta subunit expressed in an *E. coli* cell-free coupled transcription-translation system. To reveal immunoreactive nascent chains, the authors subjected RNCs to immunoadsorption with antibodies (either the conformation-dependent monoclonal antibody or one of two control antibodies) coupled to Sepharose beads [86]. The results allowed the authors to conclude that an immunoreactive folded intermediate that exhibits local structural features of the native state is formed early during tryptophan

synthase beta subunit synthesis, even before the appearance (from the ribosome tunnel) of the entire ~21 kDa N-terminal structural domain [86]. However, it was subsequently found that the antibody used in this study could itself drive formation of the epitope and was not truly conformational [87]. This highlights the need for extensive tests of conformational antibodies before they are used to monitor co-translational folding. Nevertheless, this approach has been widely used to study co-translational folding of several proteins, including MS2 phage coat protein [88], bacteriophage P22 tailspike protein [89–91], Influenza hemagglutinin [83,85], NF-kappaB1 [39], and others. One particular interesting finding from the use of conformational antibodies was that a ribosome-bound phage P22 nascent chain can adopt conformations dissimilar from early *in vitro* refolding intermediates, thus suggesting that co-translational folding pathway and *in vitro* refolding pathway may follow different routes [90,91].

3.4. Limited proteolysis

Folding of growing nascent chains into compact structures has been widely explored by proteolytic digestion [32–34,48,49,91–93]. Folding is usually assayed by partial digestion of ribosome-attached nascent chains with proteinase K, trypsin, etc. For example, Netzer and Hartl [33] investigated the synthesis and folding of a fusion protein consisting of two monomeric, single-domain cytosolic proteins, 21 kDa human H-Ras and 20 kDa mouse dihydrofolate reductase (DHFR). They demonstrated that formation of a protease-resistant Ras domain occurs (both in a eukaryotic *in vitro* translation system and in the cytosol of living cells) before the full-length fusion protein is synthesized, thus indicating that the Ras domain folds co-translationally [33]. Later, Frydman and co-authors [34] showed that proteinase K treatment of *in vitro* translation reactions directing synthesis of 62 kDa firefly luciferase resulted in formation of a characteristic 22 kDa protease-resistant N-terminal luciferase fragment that could be observed after 8 min of translation, well before completion of the synthesis of the full-length polypeptide (~12 min). The authors suggested that co-translational formation of the N-terminal subdomain may provide a scaffold for further folding, thereby preventing formation of kinetic traps and facilitating rapid *in vivo* folding [34]. Partial proteolytic digestion has also been used to assay co-translational folding of bacteriophage P22 tailspike protein [91], *E. coli* OmpR [33], SufI [92] and the N-terminal domain of N5-glutaminemethyltransferase (HemK) [48], cystic fibrosis transmembrane conductance regulator (CFTR) [93], gamma-B crystallin [49] and other proteins.

3.5. Formation of oligomeric complexes

Co-translational formation of oligomeric complexes has been viewed as an indication that the protein surfaces/conformations responsible for intersubunit interactions/contacts have been properly formed within the nascent chains. Co-translational oligomerization was originally demonstrated for tetrameric *E. coli* β-galactosidase [25,27] and has also been shown to occur during i) formation of enzymatically active bacterial luciferase, an αβ heterodimer [69–71], ii) trimerization of reovirus cell attachment protein [37], iii) dimerization of p53 [94], iv) assembly of mature NF-kappaB1 [39], v) heteromeric assembly of human ether-à-go-go-related gene (hERG) 1a/1b channel protein [95], and production of other multi-subunit proteins as well (reviewed in [96,97]). Interaction of nascent chains with chaperones during translation can be also viewed as an indication of proper co-translational folding [10,11,96,97]. Co-translational assembly can thus lead to formation of homo- or hetero-oligomeric complexes and may involve cis- and/or trans-mechanisms, whereby interacting subunits are translated from either the same or different mRNAs. A recent study by Bukau's and Kramer's group [71] deserves special mention in this regard. This elegant study used a combination of *in vivo* and *in situ* approaches including FRET, luciferase activity measurements and selective ribosome profiling (SeRP) to uncover nascent subunit interactions during luciferase complex assembly in *E. coli* cells. To express the LuxA (α) and LuxB (β) subunits of the bacterial *Vibrio harveyi* heterodimeric luciferase protein and study their *in vivo* assembly, the authors fused genes encoding monomeric variant forms of enhanced yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP) to the 5′ end of each lux gene and integrated artificial lux operons into the *E. coli* chromosome at distinct sites [71]. Four different bacterial strains, each with two artificial operons carrying different tag configurations of the yfp/cfp-luxA and yfp/cfp-luxB fusion genes were created [71]. FRET and luminescence measurements revealed that the organization of the LuxA and LuxB subunits in the operon is critically important for their co-translational assembly into an active enzyme complex. Association between the subunits was demonstrated to occur as they are being synthesized on ribosomes, with the organization of the genes in the operon greatly affecting subunit interactions and assembly. These results illustrate a fundamental co-translational mechanism that ensures effective assembly of protein complexes *in vivo* [71].

3.6. Ligand binding

The ability of co-factors and ligands (such as heme) to specifically bind to growing polypeptide chains has been viewed as an indication that a binding-competent conformation has been achieved [35,36]. Our group used heme binding to probe co-translational folding of the α-globin chain (which, in contrast to β-globin, does not give rise to oligomeric structures) [36]. Using *in vitro* translation reactions performed in the presence of [^{3}H]hemin and [^{35}S]methionine together with sucrose gradient centrifugation and puromycin treatment, we showed that ribosome-bound α-globin chains are capable of efficient heme binding [36]. In addition, we found that incomplete α-globin nascent chains of 140, 100, and 86 amino acid residues produced from truncated/non-stop mRNAs are capable of co-translational heme binding, indicating that a structure that allows for heme binding in the nascent chain is achieved prior to the completion of α-globin synthesis. These results provide strong support for co-translational folding of the α-globin molecule [36]. Similarly, studies of the biosynthesis of protein D1 of the membrane-bound chloroplast reaction center demonstrated co-translational binding of chlorophyll to incomplete D1 molecules [98,99]. More recently, ligand binding was used to probe co-translational folding and for pharmacological characterization of beta1-adrenergic receptor [100]. It couldn't be excluded however that ligand binding may promote the formation of the proper tertiary structure within the growing polypeptide on the ribosome, yet, this doesn't rule out and exclude the possibility of co-translation folding.

3.7. NMR spectroscopy

NMR spectroscopy is able to provide atomic-resolution information for ribosome-bound nascent chains of various lengths (usually not exceeding ~250 amino acids, the regular limit for NMR) [42]. While NMR has the potential to provide important structural information on folding of nascent chains during translation, application of this technique in relation to co-translational protein folding is complicated by a large number of technical challenges including the need for uniformly labeled, homogeneous, stalled RNCs in sufficient concentrations (~10 μM) [42,101]. In 2007, the first structure of a ribosome attached nascent chain complex was determined using solution-state NMR spectroscopy [40]. This was achieved using selectively labeled ^{13}C/^{15}N nascent chains of a

C-terminally truncated immunoglobulin (Ig2) protein harboring a full-length N-terminal domain (NTD) and a truncated C-terminal domain (CTD). The labeled chains were produced in an *E. coli* cell-free translation system from a non-stop mRNA and purified using sucrose density gradient centrifugation [40]. This study showed that NMR spectra of remarkable quality can be obtained for a nascent polypeptide chain attached to the ribosome and, specifically, that the Ig2 NTD is folded within the RNC to its fully native state despite retaining some interactions with the ribosome surface [40]. Later, a strategy employing a SecM stalling motif for preparation and isolation of isotopically-labeled RNCs suitable for NMR studies in *E. coli* was developed [41]. While still having a substantial drawback (time required for preparation, isolation and analysis of RNCs), a significant advantage of NMR is its ability to provide direct atomic resolution information about the structure of ribosome attached nascent chains [42,101]. NMR analysis of RNCs with nascent chains of different lengths provided successive snapshots along the co-translational folding pathway and allowed monitoring of both unfolded and folded conformations within nascent chains of different lengths [102]. Dynamic nuclear polarization (DNP) magic-angle spinning (MAS) NMR has further advanced the field by allowing analysis of the structure of the signal sequence of disulfide oxidoreductase A (DsbA) inside the ribosome tunnel [103].

3.8. Cryo-electron microscopy (cryo-EM)

Cryo-electron microscopy has played an instrumental role in the study of ribosome structure and the process of translation [45,104]. However, until recently, the resolution attainable for cryo-EM structures was limited to about 7 Å in the best cases [104]. Nevertheless, even early studies that used cryo-EM to compare the structures of free ribosomes with stalled steady-state RNCs (produced in cell-free synthesis reactions from non-stop mRNAs) demonstrated that segments of nascent polypeptide chains located inside the ribosomal tunnel (in its vestibule region) might exist in "a rudimentary globular conformation" [105]. Subsequent cryo-EM studies, primarily by Beckmann's and Wilson's groups, enabled direct visualization of the density of helices inside the tunnel [43,44,79 and Ref. therein]. However, all of these analyses had the same major drawback (time required for preparation, isolation and analysis of RNCs) as the NMR measurements described above and thus couldn't completely exclude the possibility that folding occurred during the RNC isolation steps rather than during protein synthesis. While time-resolved cryo-EM [106] could potentially allow visualization of co-translational intermediates as they form during translation, application of this technique for co-translational folding studies remains challenging at the present time. Nevertheless, the improved (~3 Å) resolution that became available with advances in development of direct electron detector cameras (direct detectors) [104] bears great potential for future co-translational protein folding studies at the atomic level, although heterogeneity of RNC samples will remain a substantial obstacle for cryo-EM image processing and 3D-reconstruction.

3.9. Atomic force microscopy (AFM)

Atomic force microscopy (AFM), also known as scanning-force microscopy (SFM), is used to study materials by scanning over the surface with a very sharp tip [107]. AFM has evolved from a basic raw/morphological imaging technique to a powerful atomic resolution approach that, similar to cryo-EM, allows investigation of biological samples at atomic resolution without labeling them [107]. This technique was recently used to determine the structural features of the large (132 kDa) membrane binding domain of ankyrin-R polypeptide attached to the ribosome [108]. SecM-stalled RNCs were produced *in vitro* in a reconstituted cell-free system free of chaperones, purified, and subjected to AFM. This captured the solenoid structure characteristic of the native ankyrin-R alpha horseshoe fold [108]. The authors suggested that AFM may be valuable for visualization of correctly folded large nascent polypeptide chains at submolecular resolution, but the technique would not be able to avoid the same major drawbacks as NMR and cryo-EM. While the advent of fast AFM [109] could potentially allow visualization of co-translational folding intermediates in real time, application of this technique for co-translational studies is far from practical at present time.

3.10. Single-molecule force spectroscopy

Single-molecule force spectroscopy (SMFS) is a powerful tool for studying protein folding [110,111]. In SMFS experiments, structural changes are monitored in molecules subjected to controlled forces using optical tweezers, magnetic tweezers and/or atomic force microscopy [110,111]. This technique was recently used to monitor folding transitions in ribosome-bound nascent chains [112–114]. For this application, the force is applied between the nascent chain and the ribosome and monitored using optical tweezers [113] (Table 1). Because the force acts locally, it is possible to selectively perturb the stability of ribosome-bound polypeptides without disrupting the structural integrity of the ribosome [113]. These types of experiments provided evidence supporting the possibility that the ribosome not only decodes mRNAs, but also actively promotes efficient *de novo* folding of nascent chains into their native state [112–114]. For example, using SMFS, Kaiser et al. [112] demonstrated that truncated T4 lysozyme polypeptides misfold and aggregate when free in solution, but remain folding-competent when they are synthesized in a reconstituted *in vitro* translation system and remain attached to the ribosome [112]. Tinoco Jr. and Bustamante and their team also used this approach to show that folding of a nascent globular protein domain can generate a pulling force capable of modulating elongation by acting on SecM-arrested ribosomes containing peptidyl-tRNA stably bound in the A site [115]. The authors postulated that the pulling force along the nascent chain could constitute an important feedback mechanism to tune elongation and folding of the growing nascent chains [115]. Dual-trap optical tweezers have been applied to observe and compare the co-translational folding of several individual nascent proteins (two globular ones; GFP variant Emerald (GFPem), and dihydrofolate reductase (DHFR) and one intrinsically disordered protein, hTau40) held at various constant forces [116].

3.11. Fluorescence anisotropy/dynamic fluorescence depolarization

Fluorescence anisotropy [117] is an approach that can provide direct information on the local dynamics of ribosome-attached nascent proteins [50–52] and thus is a valuable addition to the array of tools available to study co-translational protein folding [52]. Application of the version of this technique known as dynamic fluorescence depolarization led to identification of specific local motions in ribosome-bound nascent chain complexes (RNCs) that were believed to indicate formation of independent nascent protein structures on the ribosome [50–52]. Use of this method requires generation of homogeneous, stable, stalled, fluorescently-labeled RNCs, which can be obtained in reconstituted *in vitro* translation systems with the use of ribosome-stalling sequences or non-stop mRNAs [52 and Ref. therein]. RNC labeling is usually achieved by including fluorescently-labeled aminoacyl tRNAs in the *in vitro* translation system (typically derivatives of Met-tRNA^{fMet} in the case of *E. coli* systems), which results in N-end labeling of nascent chains. Labeled RNCs are purified via sucrose density gradient centrifugation and/or affinity/gel filtration chromatography techniques [52]. N-terminal

GFP-fusions are generally not suitable for anisotropy studies due the large size of the GFP moiety relative to the nascent chains [52]. One set of time-resolved fluorescence anisotropy experiments identified peculiar nanosecond-scale motions within relatively long apomyoglobin nascent chains (>89 amino acid residues in length), but not within the short nascent chains (16–35 residues) that were buried inside the ribosomal tunnel [50]. Nanosecond-scale motions are indicative of the presence of a rigid structure or a partially folded species. It should be noted that previous study of the closely related α-globin protein suggested that ribosome-bound α-globin nascent chains 86 amino acids in length can acquire a spatial structure that allows interaction of the nascent chain with the heme group during protein synthesis [36]. Fluorescence anisotropy experiments also demonstrated that the dynamics of the nascent chains are strongly influenced by the presence of the ribosome surface (and its overall negative charge) [51]. Many interesting questions can be addressed through use of steady-state and time-resolved anisotropy (e.g., chain behavior during the release of nascent proteins from ribosomes and/or the influence of chaperones on chain dynamics [52]). However, analysis of data in both steady-state and time-resolved anisotropy experiments is not trival and may be complicated by heterogeneity in the RNC populations (e.g., RNCs bound and unbound to chaperones) and other parameters of the system [52].

3.12. Fluorescence Resonance energy transfer (FRET)

Incorporation of two fluorescent tags into growing polypeptide chains allows both steady-state and real-time measurements of co-translational protein folding using fluorescence resonance energy transfer (FRET) [18,46–49]. FRET occurs when an excited donor dye is sufficiently close to an appropriate acceptor dye for the excited state energy of the donor to be transferred to the acceptor without emission of light. FRET measurements reflect the efficiency of the transfer of energy between the donor and the acceptor, which is directly correlated with the distance between the two fluorophores. Thus, changes in nascent chain conformations can be monitored using steady-state and time-resolved FRET measurements [18,47]. A major advantage of this technique is its extreme sensitivity. Reliable measurements can be made with samples containing just a few nM of the respective fluorophores [18,47].

Incorporation of donor and acceptor fluorophores into nascent chains for FRET experiments can be achieved via several approaches [18]. One method uses chemically modified aminoacyl-tRNAs (aa-tRNAs) to incorporate non-natural amino acids carrying a probe into nascent chains. Fluorescent probes such as 4,4-difluoro-4-bora-3a, 4a-diaza-s-indacene (BODIPY) and its derivatives can be attached to Met-tRNA^{fMet}, Lys-tRNA^{Lys}, or Cys-tRNA^{Cys} tRNAs [18,64]. Pioneered by Arthur Johnson [46,47], this approach was originally used to demonstrate that a transmembrane sequence within the nascent chain of a membrane protein likely folds into a compact alpha-helical conformation inside the ribosome tunnel and remains folded as the sequence moves through a membrane-bound ribosome into the translocon [46]. In this study, N$^{\varepsilon}$-(BODIPY FL)-Lys-tRNA^{Lys} (εBOF-Lys-tRNA^{Lys}) was used to incorporate the donor fluorophore at a lysine codon in the mRNA and N$^{\varepsilon}$-(BODIPY)-Lys-tRNA^{amb} (ϵBOP-Lys-tRNA^{amb}) was used to incorporate the acceptor fluorophore at an amber stop codon (with both the donor and acceptor tags being on lysine residues) [46]. It should be noted, however, that not every lysine and amber codon will incorporate a fluorescent amino acid due to competition with endogeneous unmodified Lys-tRNAs and termination factors, respectively; thus, only a fraction of all nascent chains will be labeled. To ensure that nascent chains with a donor will also contain an acceptor, the amber stop codon (that is poised to incorporate the acceptor fluorophore) is usually positioned before the codon that will drive incorporation of the donor fluorophore [46,47]. Thus, FRET will only take place in nascent chains that are translated beyond the amber codon (due to incorporation of the acceptor) and will further incorporate the donor; nascent chains terminated through the action of endogenous factors will contain no fluorophores and will thus be "invisible" during FRET analysis. In the original study by Woolhead et al. [46], RNCs with nascent protein chains of a defined length were prepared by translating non-stop mRNAs in a wheat germ extract cell-free translation system. Fluorescence measurements were performed on steady-state samples. Modified Met-tRNA^{fMet}, Lys-tRNA^{Lys}, or Cys-tRNA^{Cys} were further successfully used by our group for rapid kinetic experiments aimed at monitoring translation and co-translational protein folding in real-time [48,49]. These experiments demonstrated that co-translational folding of a protein that folds autonomously and rapidly in solution may proceed through formation of a non-native but compact conformation inside the ribosome tunnel, followed by rapid rearrangement into a native-like structure immediately after the entire protein emerges from the ribosome [48]. FRET using BOP-Met-tRNA^{fMet} and BOF-Cys-tRNA^{amb} donor/acceptor pairs was also used by us to demonstrate that synonymous codon usage modulates the kinetics of co-translational folding [49]. In this study, we used a fully reconstituted single-turnover *in vitro* translation system lacking translation termination factors to monitor co-translational folding of gamma-B crystallin, a two domain mammalian eye-lens protein that was produced from non-stop mRNAs. By comparing two synonymous codon variants (e.g., protein variants with identical amino acid sequences encoded by different "synonymous" codons), we showed that rates of translation (which differ for synonymous codon variants based on the availability of the respective tRNAs) affect the kinetics of co-translational folding of protein domains [49].

A combination of a small fluorophore incorporated via a modified aa-tRNA and a large fluorophore (like GFP) can be also used for FRET studies. Skach and colleagues [118] utilized a donor fluorophore, CFP, that was fused to the N-terminus of the first nucleotide-binding domain (NBD1) of the cystic fibrosis transmembrane conductance regulator (CFTR), and a small acceptor dye that was incorporated into the nascent chain via 7-nitro benz-2-oxa-1,3-diazol-Lys-tRNA^{amb}. FRET measurements of sequential stably arrested ribosome-bound nascent chains revealed early co-translational folding intermediates and delineated the CFTR co-translational folding pathway [118].

Finally, a FRET donor/acceptor pair consisting entirely of large protein fluorophores like CFP and YFP can be used. As mentioned above, CFP in combination with YFP was used to monitor co-translational assembly of heterodimeric bacterial luciferase into an active enzyme complex *in vivo* [71]. Another use of the YFP/CFP pair was by Clark and colleagues [119], who took advantage of the so-called bimolecular fluorescence complementation (BiFC) assay [120,121] to design a fluorescent protein consisting of three half-domains, where the N- and C-terminal half-domains compete each other to interact with the central half-domain. Using a cellular (*E. coli* expression) system and FRET between the YFP/CFP labels, they demonstrated that the rate at which a nascent protein emerges from the ribosome and the vectorial appearance of the nascent chain during translation can specify the final folded conformation of a protein [119].

3.13. Cysteine accessibility assay and other approaches

Strategies based on monitoring cysteine accessibility have also been developed to address questions related to co-translational protein folding. Carol Deutsch [121,122] combined pegylation (i.e., mass tagging a peptide with polyethylene glycol maleimide (PEG-MAL, 5 kDa)) and a cysteine accessibility assay to probe the accessibility of engineered cysteines introduced into growing

nascent chains of various lengths and composition/amino acid sequence. The cysteine accessibility assay is based on the postulation that the length of a stretch of nascent chain that can be located within the ribosome tunnel at a given time depends upon its conformation. For example, formation of a helix inside the tunnel will produce a more compact nascent peptide and allow a greater length of the chain to be in the tunnel. In this case, a border cysteine residue will be retracted inside the tunnel, so that it will become inaccessible to pegylation, while the same cysteine in a peptide of a similar length in an extended conformation will be available for modification. The cysteine accessibility method was used to demonstrate that the ribosome tunnel can indeed accommodate an alpha-helix, and that the formation of a compact structure depends on its location inside the tunnel [121–123]. Furthermore, a combination of cysteine accessibility and cross-linking assays showed that intramolecular tertiary interactions may occur before the nascent peptide has fully emerged from the ribosomal exit tunnel [124–126]. Compaction of the nascent peptide inside the ribosome has also been revealed using other read-outs including protease protection, enzymatic activity of the nascent protein, antibody binding, glycosylation [see 121–123 and Ref. therein], cryo-EM [43–45], and FRET [46].

In addition to all of the methods described above and summarized in Table 1, several other approaches are available to researchers studying co-translational protein folding. For example, release of SecM-arrested peptides can serve as an intrinsic sensor of the force generated as a result of nascent chain folding. This strategy was used to study DHFR folding in the presence/absence of the chaperones trigger factor (TF) and GroEL/ES [127]. It was found that DHFR folds into its native structure only when it fully emerges from the ribosome tunnel and that chaperones (TF and GroEL) substantially affect the folding process and reduce the force generated by the nascent chain [127].

4. Summary and outlook

The brief overview of the arsenal of available methods provided above reflects the remarkable progress that has been achieved in recent years in studies of protein folding [8–18]. Many advances in the field have come about due to the powerful new approaches that have been developed and applied to questions surrounding co-translational folding (e.g., NMR, cryo-EM, fluorescence spectroscopy techniques). However, even traditional techniques such as limited proteolysis remain useful [48,49]. While the concept of co-translational protein folding is supported by extensive experimental evidence and has become widely accepted, there remain pressing questions in the field to be addressed in future studies. These include: What are the exact structures formed during co-translational folding and when do they form? What structural transitions/conformational changes do these structures undergo to achieve the native state of the protein? How do translation elongation kinetics impact formation of these structures? Obtaining unequivocal answers to these questions will require further development and application of real-time approaches (such as FRET) and, ideally, single molecule experiments. Ultimately, such new data will allow us to further develop a unifying concept of protein folding.

Conflict of interests

The author declares no conflict of interests.

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