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Co-translational protein folding: progress and methods

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Proteins are synthesized as linear polymers and have to fold into their native structure to fulfil various functions in the cell. Folding can start co-translationally when the emerging peptide is still attached to the ribosome and is guided by the environment of the polypeptide exit tunnel and the kinetics of translation. Major questions are: When does co-translational folding begin? What is the role of the ribosome in guiding the nascent peptide towards its native structure? How does translation elongation kinetics modulate protein folding? Here we suggest how novel structural and biophysical approaches can help to probe the interplay between the ribosome and the emerging peptide and present future challenges in understanding co-translational folding.

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Introduction

To carry out their various functions in the cell, proteins must fold into their native three-dimensional structures. The growing polypeptide chain on the ribosome has to navigate through a complex energy landscape which is shaped not only by the sequence of the emerging protein, but also by the ribosome itself and by proteins that interact with the newly synthesized protein. Rapid and accurate protein folding is essential for cell function, as misfolding leads to the loss or alteration of protein function, proteome disbalance and increased energetic costs of protein re-folding and degradation. Misfolding of proteins is the source of many diseases such as Alzheimer's, Parkinson's and other neurodegenerative diseases, as well as of cancer and aging. The aim of this review is to summarize what is known about co-translational protein folding and discuss experimental approaches that will be important in future research. We will concentrate on four major questions: When does the protein emerging from the ribosome start to fold? How does the ribosome affect the folding trajectory? What is the link between the kinetics of translation elongation and protein folding? What are the emerging technologies to probe co-translational folding? Other aspects of co-translational and post-translational protein folding in the cell, including the effects of chaperons, can be found in recent reviews [1–3].

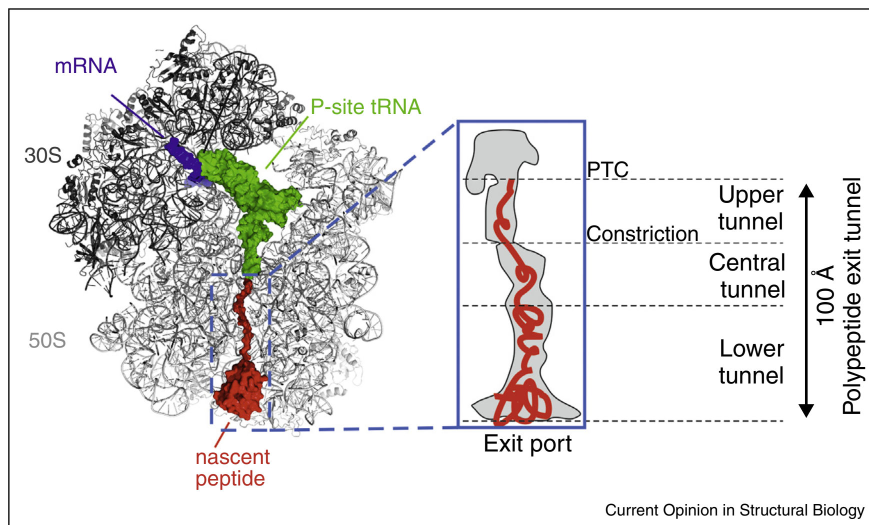
When does a nascent peptide begin to fold?

During protein synthesis, the emerging (nascent) peptide travels through the polypeptide exit tunnel of the ribosome (Figure 1), which has a length of about 100 Å and covers about 30–40 amino acids of the nascent peptide in an unfolded, fully-extended conformation. The width of the tunnel does not permit formation of large tertiary structure elements due to space limitations which preclude long-range interactions that are necessary for the cooperative folding of larger domains. Nevertheless, smaller structures can form within the tunnel, for example, compacted non-native states, α-helices, hairpins or even small α-helical domains. Formation of larger tertiary structure elements, such as domain folding, can take place when the protein emerges from the peptide exit tunnel of the ribosome ([4] and references therein). One general observation coming from these experiments is that the fundamental principles of co-translational folding appear similar in the cell and in reconstituted *in vitro* systems. In particular, this pertains to the propensity of nascent peptides to fold within the tunnel and the steering effect of the ribosome on folding. This certainly does not exclude the possibility that folding of some proteins is altered by the presence of cellular components that are absent in the *in vitro* assays used so far. However, the very good agreement between the results of *in vivo* and *in vitro* experiments suggests that the propensity for co-translational folding is a robust inherent property of the translation machinery.

How does the ribosome affect nascent peptide folding?

Contrary to the folding of isolated proteins, co-translational folding is guided by the ribosome. The ribosome may contribute to folding in a number of ways. Because co-translational folding is vectorial, that is, it involves elements that emerge successively from the N-terminus to the C-terminus, and is often limited by the rate of translation, the landscape of co-translational folding may differ from that in solution [5,6]. The ribosome may stabilize folding intermediates or conformations that are not prevalent in solution [7,8••]. Retention of these non-native folding states may represent a fundamental feature of co-translational folding that prevents the chain from falling into kinetic traps, such as stably misfolded

Figure 1



Co-translational folding of peptides on the ribosome. Left panel: Structural model of the ribosome (gray) with mRNA (blue) and a tRNA (green) carrying a nascent polypeptide chain (red) that folds co-translationally. The polypeptide chain was modeled into the polypeptide exit tunnel using the structure of the *E. coli* 50S subunit with a nascent chain, PDB 3J7Z32. The structures of authentic folding intermediates are largely unknown. First examples of structured nascent peptides are a small zinc-finger domain that folds within the exit tunnel of the ribosome [27••], an immunoglobulin-like domain that folds outside the tunnel [11•], or α-helical peptides within the tunnel observed in the ribosome complexes stalled at the specialized mRNA arrest sequences that block peptide bond formation [29]. Right panel: Folding zones of the polypeptide exit tunnel of the ribosome (modified from Refs. [1,55,56]). The upper and central parts of the tunnel are separated by the constriction. Early folding occurs in the lower part of the tunnel close to the exit port [8••,27••,55].

non-native conformations that may form when only a part of a protein has been synthesized [7,8••,9]. Because emerging peptides can interact with the ribosome surface [7,10,11•,12], the ribosome may have a chaperoning effect which protects the nascent chain from misfolding, aggregation, and degradation by cellular proteases until the protein is fully synthesized and extruded from the peptide exit tunnel [7,11•,13]. The spatial proximity of ribosomes that synthesize proteins encoded in different ORFs within one operon may ensure their efficient co-translational assembly [14••].

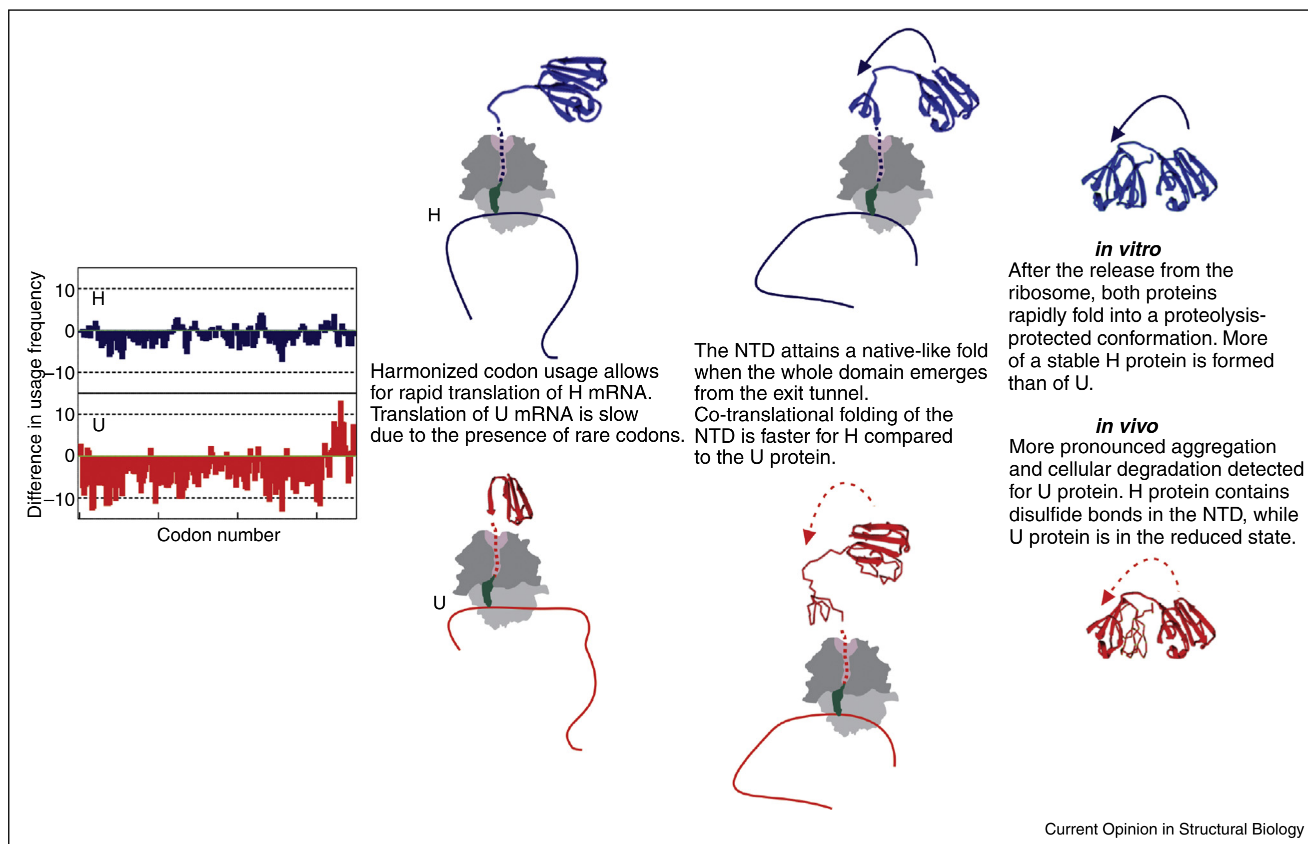
Finally, the ribosome provides a platform for ribosome-associated protein biogenesis factors that interact with the emerging nascent peptide and ensure its correct processing, folding, and targeting to its final destination in the cell. In particular, the chaperone trigger factor (TF) can affect co-translational folding. TF is the first chaperone that binds to emerging peptide chains and promotes correct folding by protecting partially folded states from forming distant interactions that may produce stably misfolded states [15].

Translation pauses and protein folding

The rate of translation is not uniform along an mRNA, with periods of rapid translation interrupted by pauses. Changes in local translation rates can influence the conformation of newly synthesized proteins [16,17]. In principle, pauses in translation may be caused by many factors, such as codon-specific rates of aminoacyl-tRNA delivery to the ribosome, the abundance of individual aminoacyl-tRNAs, codon context, or secondary structure elements in the mRNA. Bioinformatic analysis suggested that slowly-translating clusters are predominantly located downstream of domain boundaries, presumably to fine-tune translational speed with the folding fidelity of multi-domain proteins [18] or even switch folding from post-translational to co-translational [19•]. So far, most examples that link local translation velocities to protein folding are related to rare codons or to tRNA abundance [3,17,20]. Although the exact mechanism is currently debated (reviewed in [4,20]), the effect of synonymous codons on co-translational folding is well documented. Naturally occurring synonymous single-nucleotide polymorphisms (sSNPs) that alter the codon but not the amino acid specified by the codon can affect the activity and post-translational modification pattern of a protein, altering its interactions with drugs and inhibitors, sensitivity to proteases, and aggregation propensity (reviewed in [3]). In some cases, these changes are associated with diseases [21].

Our analysis of the structure and folding of γ-B crystallin has shown that synonymous mutations can change the solubility and stability of a protein in the cell and alter the conformational ensemble of the mature protein [22••] (Figure 2). The presence of rare codons in the mRNA reduces the rate of translation elongation, which results in a slower extrusion of the N-terminal domain from the exit

Figure 2



Effect of synonymous codon usage on protein folding. Two variants of mRNA coding for γ-B crystallin differ by several synonymous codon substitutions [22••]. Harmonization of the mRNA sequence (H, blue) makes synonymous codon frequencies along the mRNA almost identical in the native host (*Bos taurus*) and in the heterologous expression host (*E. coli*). Un-optimized mRNA (U, red) comprises the native sequence of bovine γ-B crystalline mRNA, but upon heterologous expression in *E. coli* the distribution of rare and abundant codons along the mRNA becomes different than in the native host due to the codon usage disparity between the two organisms. Appearance of distinct folding intermediates is linked to the speed of translation. Altered codon usage changes kinetics of translation and the folding events on the ribosome and this may lead to different final conformations of the protein (red and blue, respectively).

tunnel and slower folding of the N-terminal domain. These observations suggest that altered translation rates can result in the formation of alternative protein conformations that are retained in solution after the release of the completed protein from the ribosome. However, exactly which types of translational pauses affect co-translational protein folding, what the kinetics regimes are, and which mechanistic differences determine altered protein folding remains unclear.

Biochemical and structural approaches to protein folding

Answering outstanding questions in co-translational folding requires a combination of different experimental approaches. Early experiments relied on biochemical assays such as probing of folded state by limited proteolysis, crosslinking, monitoring gain of functional activity, interactions with antibodies, or detection of covalent modifications (reviewed in [1,23]). A combination of crosslinking, ligand binding, and immunoprecipitation with ribosome profiling can provide very detailed information as to when peptides start to fold *in vivo*. For example, ribosome profiling demonstrated that in mammalian cells protein domains acquire their native state shortly after the emergence of the entire domain from the exit tunnel [24]. More recently, von Heijne and colleagues developed an approach to probe protein folding using SecM arrest-peptide-mediated force measurements [25,26,27••]. The essence of the method is that folding of a protein that is still in contact with the ribosome should exert a pulling force on the nascent chain which should alleviate SecM-induced stalling. The method is potentially suitable to study not only large numbers of different proteins, but also the effect of chaperons [15,26,27••].

One important, poorly studied aspect of co-translational folding is the structure of ribosome-bound polypeptides.

NMR structures of ribosome-nascent chain complexes support the idea that proteins can fold as they emerge from the exit tunnel [11•,28], whereas cryo-EM showed the structures within the exit tunnel of the ribosome [27••,29]. Both NMR and cryo-EM have the potential to resolve conformational heterogeneity within the sample, which is expected if co-translational folding proceeds through multiple, non-exclusive pathways. The problem of the structural approaches is the necessity to work with stalled complexes, which may adopt conformations that are not sampled or disfavored during ongoing translation. One very exciting possibility is to use time-resolved cryo-EM with time resolution in the subseconds range [30•]. This could allow to freeze the ensembles of translating ribosomes with various peptide lengths and to derive the structures of folding intermediates by extensive computational sorting. Unfortunately, time-resolved cryo-EM — despite its great potential for a large variety of questions — is still in its infancy and its feasibility for the protein folding problem remains to be seen.

Probing nascent peptide structure by fluorescence techniques

The necessity to follow protein folding in real time has prompted several labs to search for alternative technologies, such as fluorescence ensemble kinetics and single-molecule methods. In particular, probing structural rearrangements by distance changes between reporter groups due to Förster resonance energy transfer (FRET) and photo-induced electron transfer (PET) can provide direct information about the onset of co-translational folding [8••,22••,31•]. One formidable challenge of these approaches is to engineer fluorescence reporters into the nascent polypeptide. In some cases, intrinsically fluorescent proteins can be used, in particular for *in vivo* studies. Using a designed fluorescent protein consisting of three half-domains, where the N-terminal and C-terminal half-domains compete with each other to interact with the central half-domain and where the outcome of this competition determines the fluorescence properties of the resulting folded structure, Clark and colleagues have demonstrated that the co-translational folding pathway *in vivo* differs from that of protein refolding *in vitro* [32]. Sometimes a combination of a fluorescent protein with a small acceptor dye incorporated site-specifically into the protein can be used. Such a combination was used by Skach and colleagues to delineate the pathway of co-translational folding of the first nucleotide-binding domain of the cystic fibrosis transmembrane conductance regulator [31•]. While these approaches are particularly valuable as they can be used *in vivo*, understanding nascent protein dynamics and identification of folding events within the polypeptide exit tunnel requires methods with better spatial resolution. This can be achieved by co-translational incorporation of two small organic fluorophores into the nascent chain.

Conceptually, the incorporation of two fluorescence reporters into the growing polypeptide chain is a straightforward technology. Early work showed that derivatization of the εN-lysine attached to $tRNA^{Lys}$ allows for the incorporation of fluorescent reporter groups into nascent proteins [33]. The invention of orthogonal approaches for the incorporation of non-natural amino acids *in vivo* in combination with codon reassignment strategies [34,35] opened new perspectives for the incorporation of useful fluorophores [36]. However, in practice, it is difficult to achieve high incorporation yields of fluorescence-labeled amino acids. Aromatic heterocyclic side chains can be co-translationally incorporated into peptides, but the incorporation efficiency strongly depends on the length and the chemical structure of the linker between the heterocyclic substituent and the amino acid [37–39]. Among different available reporters, fluorescent probes with the 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) core can be efficiently incorporated as FRET pairs into the nascent chain [8••,22••,38,40–42]. Importantly, BODIPY incorporation does not appear to alter the kinetics of translation or the folding of the nascent peptide [8••,22••]. Together with high extinction coefficient and quantum yield, this makes BODIPY dyes ideal for ensemble kinetic measurements of co-translational folding. For example, rapid kinetic experiments with BODIPY dyes attached to Met-$tRNA^{fMet}$, Lys-$tRNA^{Lys}$, or Cys-$tRNA^{Cys}$ allowed us to monitor translation and co-translational protein folding in parallel in the time range from milliseconds to hundreds of seconds [8••,22••].

One attractive complement to FRET measurements is PET between a suitable fluorophore and a quencher; conveniently, Trp is a strong PET quencher, which obviates the necessity to introduce two bulky dyes into the nascent chain. PET quenches fluorescence upon van der Waals contact between fluorophore and quencher and is ideally suited to study conformational rearrangements on short length scales [43,44]. As a proof-of-principle, PET between BODIPY and Trp has been successfully used not only to monitor folding [8••], but also to probe the dynamics of a large macromolecule, such as the translocon [45]. The approach has a great potential in both ensemble kinetics and single molecule format.

While very useful for ensemble measurements, BODIPY dyes are not well suited for single-molecule detection, especially in terms of photo-stability, compared to the commonly used single-molecule probes of the rhodamine and cyanine class [46]. Amino acids modified by rhodamine dyes are not well accepted by the peptidyl transferase center of the ribosome and their incorporation appears to be restricted to positions close to the N-terminus of the nascent chain [47]. Cyanine fluorophores can be successfully incorporated at internal positions of the nascent chain, but the incorporation efficiency is highly dependent on the amino acid context at the incorporation site [48]. The

experimental challenges for the incorporation of fluorescent probes suitable for single-molecule detection are the major obstacle on the way to studying co-translational protein folding by single-molecule approaches. If this hurdle is crossed, one can envisage co-translational folding visualized by single-molecule FRET and tracking techniques *in vitro* or in intact cells [49].

Nascent protein dynamics

Each time an amino acid is incorporated into the growing peptide, the peptide moves within the tunnel and can probe different conformations depending on the amino acid sequence and the local environment of the exit tunnel. Because translation appears to be slower than most folding events, folding is a quasi-equilibrium process [50]. At these conditions, the local dynamics of the nascent chain becomes particularly important. One approach to monitor chain dynamics is to measure the anisotropy of the nascent chain at different folding states. Cavagnero and colleagues studied the dynamics of nascent peptides by measuring the anisotropy decay of BODIPY FL attached to the N-terminus of apo-myoglobin (apoMb) [51,52]. The results suggest that the ribosome restricts the mobility of the polypeptide chain until it is released from the ribosome [52]. These studies pioneered the analysis of co-translational peptide dynamics; however, such experiments do not monitor intra-chain dynamics directly. Chain dynamics may be extremely rapid and occur on very short timescales down to nanoseconds [49], necessitating the use of biophysical methods with high time resolution. Moreover, folding ensembles are structurally heterogeneous, which calls for the application of single-molecule techniques. These problems can be potentially solved by a combination of single-molecule PET and fluorescence correlation spectroscopy (FCS), which can provide the tools to monitor the dynamics of individual nascent polypeptide chains down to the nanoseconds timescale. So far, these approaches have been utilizes to monitor the folding of isolated proteins [43,53,54]. In the future it should be possible to adapt these methods to monitor the dynamics of nascent chains during different stages of co-translational protein folding.

Concluding remarks and future perspectives

Recent studies not only provided insights into the mechanism of co-translational folding, but also raised new questions. For example, it remains unclear which types of protein structures/motifs/domains can fold within the ribosome, and which can adopt their native structure only after emerging from the peptide exit. What are the folding pathways of proteins with different domain topology (α-helical domain structures, β-structures, α/β-structures)? Is protein compaction a ubiquitous early folding event on the ribosome or are the early steps specific for each protein? What are the structural characteristics of the compact states ensemble? What is the link between the kinetics of translation and folding and do changes in translation velocity shape the landscape of protein folding? Answering these questions requires development of novel structural and biophysical approaches. It would be exciting to see folding of single polypeptides as they emerge from the ribosome in real time and this is a formidable goal to address.

Conflict of interest

Authors declare no conflicting interests.

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