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Thermodynamics of co-translational folding and ribosome−nascent chain interactions

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Abstract
Proteins can begin the conformational search for their native structure in parallel with biosynthesis on the ribosome, in a process termed co-translational folding. In contrast to the reversible folding of isolated domains, as a nascent chain emerges from the ribosome exit tunnel during translation the free energy landscape it explores also evolves as a function of chain length. While this presents a substantially more complex measurement problem, this review will outline the progress that has been made recently in understanding, quantitatively, the process by which a nascent chain attains its full native stability, as well as the mechanisms through which interactions with the nearby ribosome surface can perturb or modulate this process.

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Introduction

Co-translational folding is the progressive acquisition of a protein's native structure as it emerges from the ribosomal exit tunnel during translation. It is a fundamental process providing for the efficient biosynthesis of functional molecules [1] and assembly of quaternary structure [2,3••]. In many cases, co-translational folding can reduce the risk of misfolding, that is, the formation of k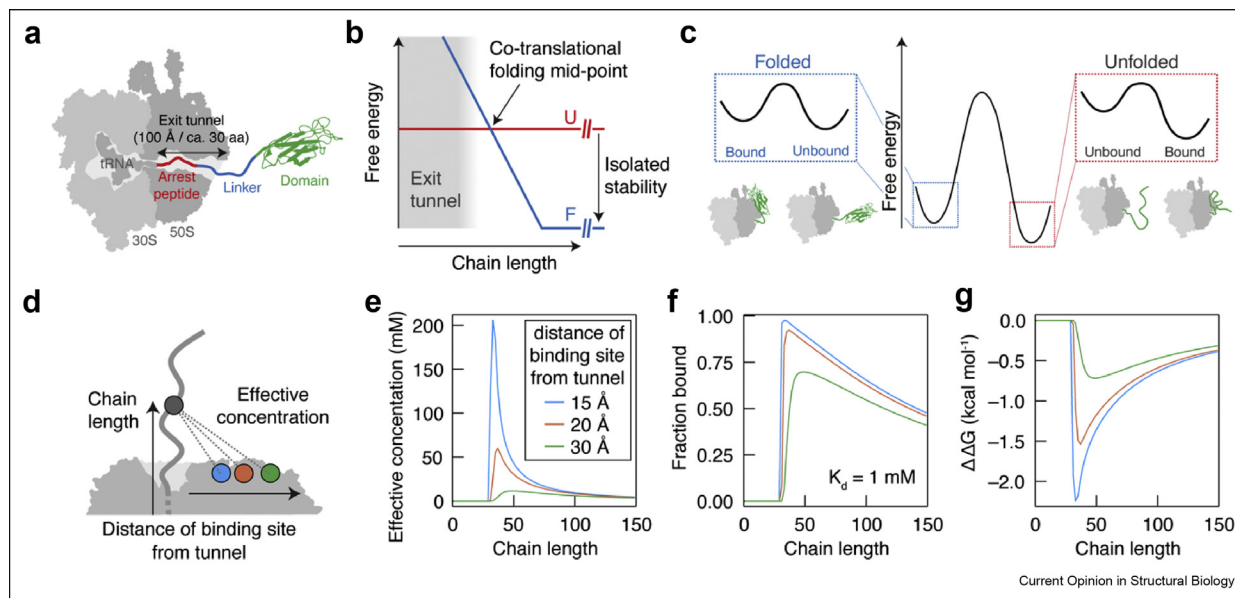inetically trapped non-native states that may be prone to further aggregation [4•,5]. Protein folding pathways have evolved to reflect the progressive synthesis of the nascent chain on the ribosome [6,7], and synonymous mutations that disrupt the rhythm of translation can lead to altered and often deleterious folding outcomes leading to misfolding disease [8−10•]. Indeed, while misfolding is a risk at any stage of a protein's lifecycle, increasing attention is now focusing on understanding this process at the earliest stages of protein biosynthesis, in variety of proteins including the cystic fibrosis transmembrane conductance regulator [10•], α_1-antitrypsin [11], huntingtin [12], human G-coupled protein GnRHR [13], and the Kv7.2 channel linked to epilepsy [14]. Biophysical studies are beginning to probe the molecular mechanisms of misfolding within translationally-arrested ribosome-nascent chain complexes (RNCs) [11,15•], and increasing evidence is emerging of sophisticated ribosome quality control pathways, including co-translational ubiquitination of translating ribosomes [16,17], and the non-canonical recruitment of release factors to terminate translation of aberrantly folded nascent chains [18].

In this review, we focus on emerging efforts to understand basic thermodynamic aspects of co-translational folding, chiefly through observations of RNCs that provide equilibrium snapshots of folding at varying polypeptide chain lengths (Figure 1a). We will also review recent measurements of interactions between emerging nascent chains and the nearby ribosome surface. Together, these results provide increasing evidence that the ribosome may not be a passive player in the co-translational folding process, but can have an intrinsic, short-range holdase activity.

Theory

The folding of isolated protein molecules is now well understood to correspond to the process of diffusion across a free energy landscape [21]. However, co-translational folding adds complexity to this picture, because the free energy surface (and indeed the dimension of the space itself) evolves with increasing polypeptide chain length [22]. To understand co-translational folding, we must therefore consider how the relative free energies of unfolded (U) and native

Figure 1

Thermodynamics of co-translational folding and the impact of ribosome−nascent chain interactions. **(a)** Co-translational folding in a translationally-arrested ribosome−nascent chain complex (RNC). **(b)** Schematic plot of the change in free energy in folded and unfolded states during translation, and upon release. **(c)** Free energy diagram illustrating the effect on a co-translational folding equilibrium of interactions of unfolded and folded states with the ribosome surface. **(d)** Illustration of the geometry of the ribosome exit tunnel and the variation in effective concentration observed for a ribosome binding site according to a residue on a disordered nascent chain. **(e)** Variation in the effective concentration of a ribosome binding site, positioned as indicated, observed by a residue along the nascent chain. Effective concentrations were estimated using a worm-like chain model of the nascent chain with a 4 Å persistence length [19]. **(f)** Estimated extent of ribosome binding for a ribosome−nascent chain interaction with a dissociation constant of 1 mM, for effective concentrations estimated in Fig. 1e. **(g)** Destabilising impact of an interaction between an unfolded nascent chain and the ribosome surface on the observed co-translational folding equilibrium [20••].

(N) states, as well as any intermediates, vary as a function of polypeptide chain length (Figure 1b).

Following synthesis at the peptidyl transference centre (PTC), a nascent polypeptide must pass through the ribosomal exit tunnel, a confined environment varying between 10 and 20 Å in diameter and ca. 100 Å in length, enclosing approximately 30−40 amino acids, which restricts formation of all but the simplest elements of secondary and tertiary structure [23,24•]. As the nascent chain emerges into the external environment, steric restrictions become reduced and co-translational folding to the native state may therefore become energetically favourable. This threshold can be represented by the co-translational folding mid-point, the chain length at which unfolded and native states are isoenergetic and therefore equally populated at equilibrium (Figure 1b). However, the onset of folding may be affected by more than steric factors: in particular, due to the proximity of the tethered nascent chain to the parent ribosome, interactions between the nascent chain and the ribosome surface may also be expected to perturb the energetics (and kinetics) of co-translational folding through a mass-action effect (Figure 1c).

It is instructive to estimate the potential effect of these interactions on the co-translational folding process. Assuming that the interaction of a segment of an emerging nascent chain occurs with a single, well-defined site on the ribosome surface, we may estimate the effective concentration of this site, as encountered by the NC segment (Figure 1d). This is defined as the concentration of ribosomes required to achieve the same extent of intermolecular interactions as within the tethered complex, and can be estimated using simple polymer physics models of the nascent chain [19]. The effective concentration depends on the distance of the binding site from the end of the ribosomal exit tunnel, and varies along the length of the nascent chain, but can be on the order of 10−100 mM, significantly higher than bulk solution (Figure 1e) [25]. Therefore, even for nominally weak interactions with dissociation constants (K_d) on the order of 1 mM, NC segments may still be strongly (>95%) bound to the ribosome surface (Figure 1f).

The impact of such interactions on the observed folding equilibrium may be determined using a simple thermodynamic model (Figure 1b): the apparent change in

stability due to an interaction of the unfolded state is $\Delta\Delta G = RT \ln(1 - p_B)$, where p_B is the fraction bound to the ribosome, T is the temperature and R is the gas constant [20••]. From this, we observe that apparently weak interactions (in isolation) may destabilise co-translational folding by ca. 1–3 kcal mol^{-1} (Figure 1g). The effect is particularly significant for segments that have only just emerged beyond the ribosomal exit tunnel, when effective concentrations are maximal, but some perturbations are predicted to persist during the translation of over one hundred further residues. Moreover, by increasing the total free energy barrier to be overcome, interactions will also inhibit folding kinetics (Figure 1b) [26]. For example, a 90% interaction is expected to decrease the rate of folding by an order of magnitude, and given that co-translational folding is fundamentally a non-equilibrium process [22], such kinetic effects may be particularly significant in determining the outcome of co-translational folding processes.

In summary, here we have provided simple theoretical arguments that by inhibiting folding, interactions between unfolded nascent chains and the ribosome surface might give rise to an intrinsic, short-range holdase effect. We speculate that this may provide time for additional residues to emerge from the ribosomal exit tunnel prior to folding, or for the folding of N-terminal regions to be completed. In the following sections, we will move beyond these theoretical arguments and review recent experimental efforts to characterise both the thermodynamics of co-translational folding, and the extent of ribosome–nascent chain interactions.

Thermodynamics

The folding of isolated protein domains can readily be observed using a variety of methods sensitive to changes in protein structure, such as circular dichroism spectroscopy or intrinsic fluorescence, and the thermodynamic stability can then be determined by measuring how the relative folded and unfolded populations are perturbed by a denaturant such as urea [27]. Fundamentally, measurements must be performed under conditions where both unfolded (U) and folded (F) states are substantially populated, in order to determine the stability $\Delta G_{U-F} = RT \ln([F]/[U])$, which may then be extrapolated back to native conditions.
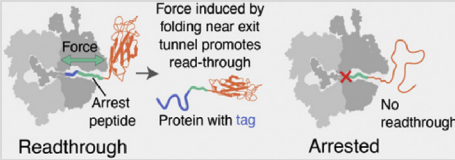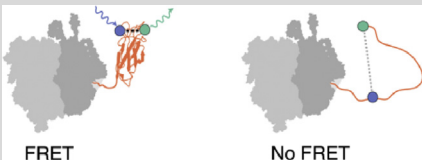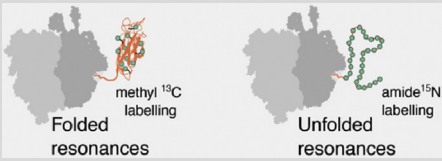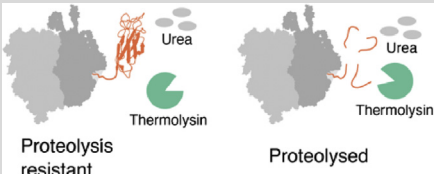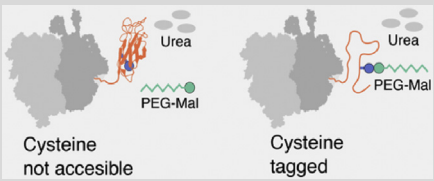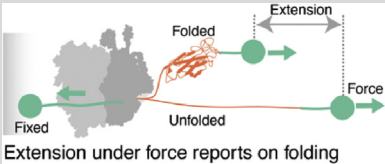
By contrast, measuring the stability of nascent chains during translation introduces three major technical challenges. Firstly, homogenous samples of RNCs must be prepared in which translation is arrested at a specific chain length, typically using the natural arrest of constructs lacking stop codons, or by incorporation of arrest peptides such as SecM [28,29]. Particular care must be taken that samples do not contain released nascent chains that might interfere with measurements of the RNC. Secondly, a detection technique must be devised

to selectively observe the nascent chain against the background of ribosomal proteins and rRNA. Lastly, perturbations must be carefully selected so as not to disrupt the integrity of the core ribosome particle.

It has been determined that RNCs can remain stable up to concentrations of ca. 3 M urea, which is adequate to at least partially unfold many nascent chains [20••,30••]. Alternatively, destabilised protein variants can be used to promote unfolding transitions within this range [30••]. However, perhaps the most natural approach is to vary the polypeptide chain length itself, allowing measurements of stability to be performed in the vicinity of the co-translational folding midpoint, at which both folded and unfolded states are populated [25].

By comparing the stability of emerging nascent chains with that of the isolated protein, one can begin to probe how the ribosome may alter the thermodynamic stability. At chain lengths where part of the domain may still be sequestered within the exit tunnel, distinguishing the impact of steric exclusion from additional effects is a particular challenge. Cryo-electron microscopy is increasingly being applied to study the relatively constrained conformation of nascent chains within the exit tunnel, which also helps to understand the positioning the remainder of the nascent chain beyond the exit tunnel [31,32]. C-terminal truncations of the isolated domain can also be used to mimic the chain length-dependence of folding, providing a baseline to interpret the effects from the ribosome [33] or, in the case of the FLN5 filamin domain, to identify potential intermediate species that may be populated along the co-translational folding pathway [34•].

A variety of experimental approaches have been developed to characterise the onset and energetics of co-translational folding (Table 1). Force profile analysis is a simple approach in which folding of a translationally-arrested nascent chain in the vicinity of the ribosome exit tunnel generates sufficient mechanical force to release the arrest peptide, resulting in translation of additional residues that can be detected using gel electrophoresis [31]. Force generation has been shown to correlate with the onset of folding in the exit tunnel and with the global stability of domains [35], and so can be a straightforward method to locate co-translational folding midpoints. However, destabilising mutations often do not perturb force profiles, indicating that they are not direct measures of stability [36], and force profiles can also be affected by the topology of the domain [35], translation speed [37] and presence of folding intermediates [38•]. Nevertheless, force profile analysis can be a relatively high-throughput approach useful for comparative studies, and provides one of the only routes established thus far to probe co-translational folding within living cells [39].

Table 1

Summary of experimental approaches for the measurement of nascent chain stability.

Experimental Approach/ Readout		Advantages	Disadvantages	Ref
	Folded ⇌ Unfolded			
Force profile analysis Mass shift on gel	Readthrough — Arrested	High throughput Determination of co-translational folding mid-points Applicable in vivo	Indirect detection of co-translational folding, not a true thermodynamic measurement May be difficult to interpret	[31]
Fluorescence FRET/PET	FRET — No FRET	Sensitive, low sample amounts Can detect folding events inside exit tunnel Applicable to real-time translation	Mutagenesis required to introduce suitable pairs of fluorophores Complex setup using non-native amino acids FRET signal sensitive to conformational changes	[41,42]
NMR Resonance intensities	Folded resonances — Unfolded resonances	Quantification of folded and unfolded populations High-resolution structural information Effectively label-free	Large sample amounts Expensive isotopic labelling may be required Multiple labelling schemes required to observe folded/unfolded states	[29]
Pulse proteolysis Band intensity on gel	Proteolysis resistant — Proteolysed	Sensitive, low sample amounts Quantification of folded population vs urea concentration RNCs stable up to ca. 3 M urea	Applicable to single domains Linker sequence must not be digestible May report on local unfolding events	[30,49]
PEGylation Mass shift on gel	Cysteine not accesible — Cysteine tagged	Sensitive, low sample amounts Quantification of unfolded/folded populations vs urea concentration RNCs stable up to ca. 3 M urea	May require mutagenesis to eliminate surface cysteines and introduce internal cysteine residues May report on local accessibility instead of global folding	[20,25,54]
Force spectroscopy Extension under force	Extension under force reports on folding	Quantification of folded and unfolded populations Determination of folding kinetics Detection of intermediates and misfolding	Expensive, specialised apparatus required	[26]

If selective labelling or observation of nascent chains can be achieved, then spectroscopic methods provide powerful tools for the analysis of co-translational folding. Ongoing advances in the incorporation of fluorescent labels via non-natural amino acids [40] have enabled measurements of FRET and PET within nascent chains during real-time translation and in arrested RNCs [41–43•]. This approach has proven valuable not only in analysing the onset of folding, but also in detecting the formation of compact intermediate species along the co-translational folding pathway. Alternatively, NMR spectroscopy relies on the selective observability of flexible nascent chains against the ca. 2 MDa core ribosome, and can provide not only direct structural and thermodynamic information based on resonance intensities and chemical shifts [25,29,44,45], but also high-resolution probes of the dynamics and interactions of both folded and unfolded states (discussed further below) [20••,46,47•], including within living cells [48].

As an alternative to biophysical methods, a variety of gel-based, biochemical approaches have been developed to measure co-translational folding, using western blotting, radiography or fluorescent dyes to detect nascent chains with high sensitivity and specificity. Pulse proteolysis may be used to determine the populations of folded states resistant to degradation [49], and indeed provided one of the first direct proofs of co-translational folding, in firefly luciferase [1]. The approach has more recently been applied to RNCs equilibrated in varying concentrations of the chemical denaturant urea, yielding important, quantitative measurements of nascent chain stability that showed destabilisation of three separate systems, RNase H, DHFR and barnase, relative to the isolated domains (discussed further below) [30••]. Cysteine modification can also be employed in place of proteolysis [50,51], using a high-molecular weight polyethylene glycol (PEG)-maleimide mass tag to measure both the emergence of solvent exposed cysteine residues from the exit tunnel [25], or thermodynamic stability through the response of a buried cysteine residue to urea denaturation [20••].

Lastly, single molecule force spectroscopy is a powerful, if specialised, technique for the study of co-translational folding [26,39]. By applying force between the nascent chain and the large subunit, transitions between compact folded states and extended unfolded states can be monitored in real time, proving detailed information on both thermodynamic stability and folding kinetics. These studies have now found for both T4 lysozyme and elongation factor G (EF-G) that in close proximity to the ribosome, both thermodynamic stability and folding rates are decreased [26,52•,53•].
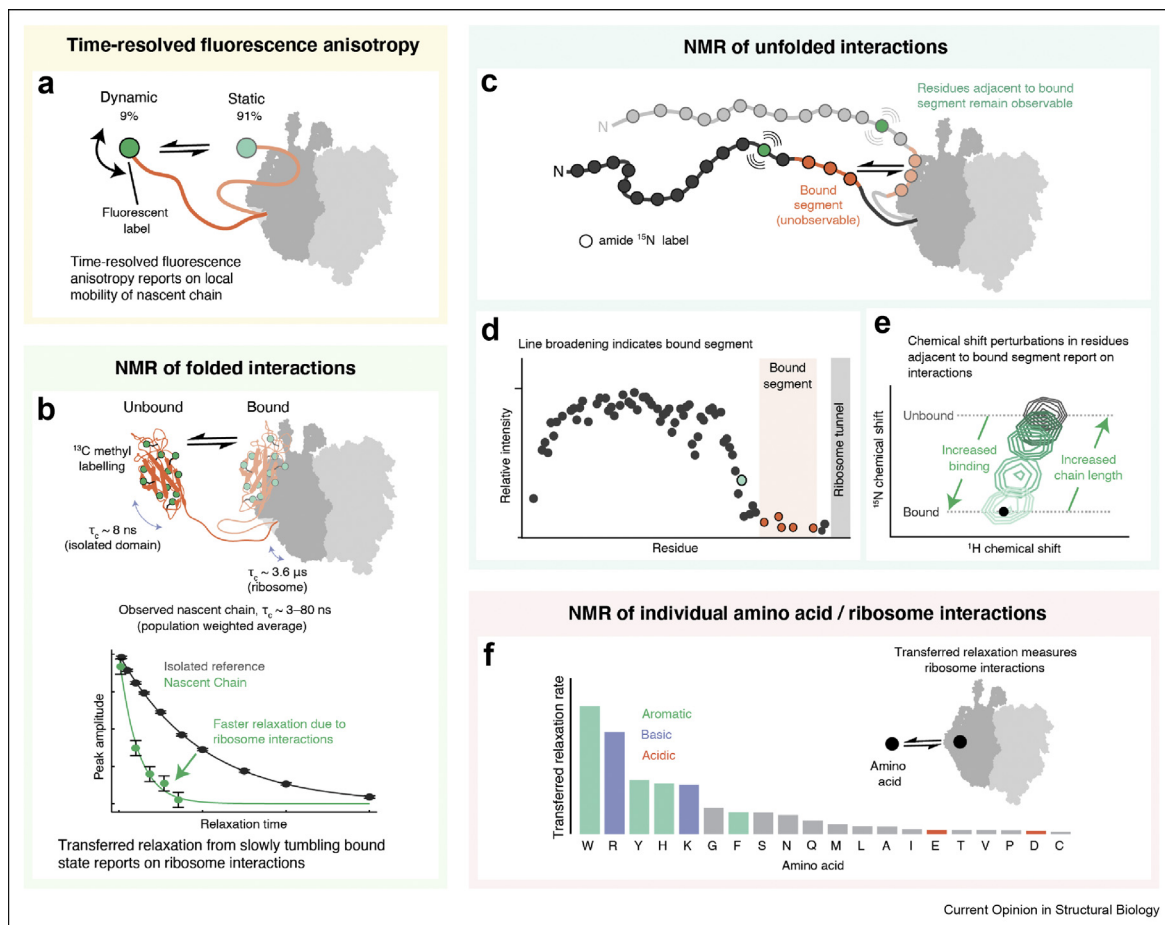
Collectively, these studies have begun to establish a pattern of behaviour, in which the onset of co-translational folding occurs ca. 30–40 residues downstream of the PTC, when the nascent polypeptide fully emerges from the confines of the ribosomal exit tunnel. However, where measured, for many systems including FLN5 [34•], T4 lysozyme [26], EF-G [52•], RNase H, DHFR and barnase [30••], thermodynamic stability was found to be reduced by up to several kcal mol^{-1} in proximity to the ribosome, until the nascent chain emerges a further 10–20 residues from the exit tunnel, corresponding to an increase in chain length of ca. 35–70 Å. This destabilisation has been associated with a decreased folding rate [26,38•] and is inversely correlated with isoelectric point [30] or reduced at high ionic strength [26]. On this basis, it has been suggested that destabilisation may arise, at least in part, through interactions between the unfolded nascent chain and the ribosome surface [25,26,30••,38•]. Therefore, the next section will review efforts to quantify such interactions and correlate them with changes in thermodynamic stability.

Nascent chain−ribosome interactions
Recent thermodynamic measurements have identified a systematic trend towards destabilisation of nascent chains close to the ribosome that has been ascribed, at least in part, to the effect of interactions between unfolded nascent chains and the ribosome surface, which presents a highly negatively charged combination of protein, RNA and counterions in the vicinity of the exit tunnel [55•,56]. However, it is important to test this mechanistic link by measuring the strength of such interactions, so that they might be compared with thermodynamic observations. By uncovering the structural basis of these interactions, we might also hope to achieve a more predictive understanding of co-translational folding, which in turn could further our understanding of other co-translational processes, such as misfolding [4•,15•,52•] and complex formation [2,3••].

As for the measurement of nascent chain stability discussed above, the quantification of interactions between nascent chains and the ribosome surface presents a number of technical challenges, and two experimental techniques have thus far proven able to quantify the extent of intramolecular ribosome−nascent chain interactions: time-resolved fluorescence anisotropy [56••−59] and NMR spectroscopy [20••,45−47•]. The former approach is based on distinguishing fluorescently labelled nascent chain populations that are static or dynamic on the ca. 5 ns timescale of the fluorescence lifetime, and in the case of the intrinsically disordered protein PIR, found substantial static populations that were inferred to be associated with the ribosome surface (Figure 2a) [57].
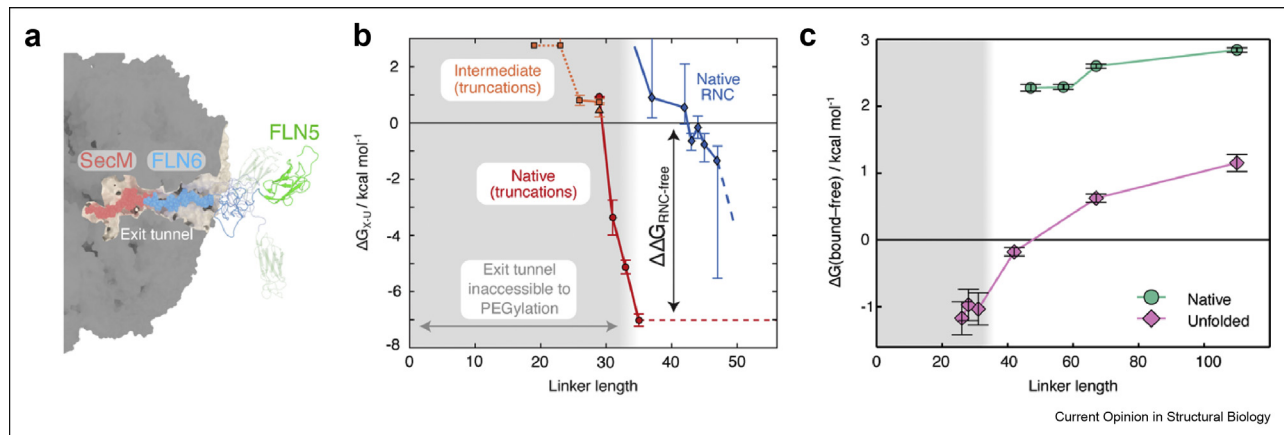
Figure 2

Experimental approaches for quantification of ribosome−nascent chain interactions. **(a)** Time-resolved fluorescence anisotropy determines populations of fluorescently-labelled nascent chains that are dynamic or static on the timescale of the fluorescence lifetime, the latter populations likely interacting strongly with the ribosome surface [57,59]. **(b)** Solution-state NMR spectroscopy of folded nascent chains using selective $^{13}CH_3$ methyl labelling. Exchange between free and bound states results in transferred relaxation from the slowly tumbling core ribosome, which can be measured and interpreted in terms of a bound population [47•]. **(c)** Solution-state NMR of unfolded nascent chains using ^{15}N labelling. **(d)** Interactions with the ribosome surface lead to intensity losses than can be used to identify the location of interacting segments [25,45]. **(e)** Chain-length-dependent chemical shift perturbations observed in residues adjacent to unobservable strongly interacting segments can be used to quantify the extent of binding [20••]. **(f)** Transferred relaxation measurements can determine the relative strength of interaction between free amino acids and purified ribosomes [20••].

NMR methods can also assess the extent of interactions through relaxation or cross-correlated relaxation measurements of mobility, in both folded and unfolded states using appropriate isotopic labelling schemes (Figure 2b−e) [20••,45−47•]. Applications of this approach are inevitably limited to weak interactions (approximately less than 5% binding) due to the signal loss associated with binding to the large, slowly tumbling ribosome, but such intensity changes can also serve as an excellent tool for identifying the location of interaction sites, particularly within disordered nascent chains (Figure 2d) [20••,25,45]. Residues adjacent to unobservable, strongly interacting segments can also be used as indirect probes of binding, by reporting on the altered chemical environment of the bound state while

retaining sufficient mobility in themselves to be observable (Figure 2e) [20••]. By varying the nascent chain length, the effective concentration of the ribosome surface and hence the extent of binding could be varied (Figure 2e), and analysis of these perturbations across multiple nascent chain sequences allowed the chemical shift of the fully bound state to be determined, and hence the extent of binding to be calculated across all lengths [20••].

Together, these methods have revealed interactions with a range of strengths, from under 1% to over 95% bound. Many studies have highlighted the role of electrostatics in determining ribosome-nascent chain interactions, through modulation of ionic strength [20••]

Figure 3

Co-translational folding of the FLN5 filamin domain. **(a)** Three conformations from a structural ensemble of the FLN5+110 RNC (comprising the FLN5 domain and a 110 aa linker composed of the subsequent FLN6 domain and SecM arrest peptide) determined by NMR chemical shift-restrained molecular dynamics simulations [25]. **(b)** Chain length-dependence of the free energy of folding for FLN5 RNCs, determined by analysis of NMR resonance intensities, compared with length-dependent measurements of the isolated domain determined by C-terminal truncation, aligned based on the number of residues obscured within the exit tunnel determined by PEGylation [34●]. **(c)** Free energy of FLN5−ribosome interactions measured for folded and unfolded states as a function of nascent chain length. Shading indicates the approximate extent of polypeptide enclosed within the exit tunnel. Adapted from [47●].

(including indirectly through its effect on nascent chain stability [26,30●●]) or mutational analysis [20●●,45,47●,57]. However, net charge alone is not sufficient to predict interactions, and aromatic residues have also been indicated to play a role [45,56●●]. Such observations are in at least qualitative agreement with a simple NMR-based measurement of the interaction of individual amino acids with unprogrammed 70S ribosomes, which were strongest for aromatic and basic residues (Figure 2f) [20●●].

The location of interaction sites on the ribosome surface is a largely unresolved question. While cryo-electron microscopy is well suited to the analysis of nascent chains within the confines of the exit tunnel, and density may sometimes be observed for folded domains located close to the end of the tunnel [23,31,32], so far longer range, more dynamic interactions have proven elusive. Instead, chemical cross-linking, using fluorescently labelled nascent chains coupled with western blotting of ribosomal proteins, may be used to map interactions at the domain level, and in the case of the intrinsically disordered protein PIR identified interactions with the L23 and L29 ribosomal proteins located close to the end of the exit tunnel [56●●]. Lastly, coarse-grained molecular dynamics simulations, potentially incorporating experimental constraints, are also a useful tool for investigating nascent chain−ribosome contacts, and despite the challenges of accurately modelling protein/RNA interactions and the effects of magnesium

counterions, remarkably good agreement with experiment has been found for predictions of co-translational folding [24,60,61] and the effect of interactions [20●●].

Case study: Co-translational folding of the FLN5 filamin domain

The above sections have outlined recent progress in analysing both co-translational folding thermodynamics and ribosome−nascent chain interactions. FLN5, the fifth immunoglobulin domain from the tandem repeat protein filamin, provides the first example of a system for which both sets of measurements have been carried out. The co-translational folding of this protein has been studied by NMR for over a decade, employing the subsequent FLN6 domain as a natural linker sequence [62,63]. NMR chemical shift-restrained molecular dynamics simulations were employed to determine an ensemble RNC structure (Figure 3a), while measurements of NMR resonance intensities allowed the stability of the nascent chain to be determined as a function of chain length (Figure 3b) [25]. A significant difference was observed between the observed stability and that estimated from an isolated C-terminal truncation model, with lengths aligned based on the measured emergence of the domain from the exit tunnel (Figure 3b) [34●]. A shallower folding transition was observed as a function of length than for the isolated truncation model, and no evidence of a putative co-translational intermediate associated with proline isomerisation could be detected within RNCs. These observations pointed to a substantial difference

between the stability of the domain in isolation and on the ribosome, $\Delta\Delta G_{\mathrm{RNC-free}}$ (Figure 3b).

To assess the contribution of ribosome–nascent chain interactions to the observed co-translational folding offset, NMR-based measurements of these interactions were developed and performed, for both folded and unfolded states in order to cover both sides of the folding equilibrium (Figure 3c). Relaxation-based measurements of the folded domain found a weak, length-dependent interaction, on the order of 5% binding or less [46,47•]. In contrast, a strong interaction of a C-terminal segment was observed within the unfolded state (Figure 2d), and analysis of chemical shift perturbations in adjacent residues (Figure 2e) showed this to correspond to up to 95% binding at short chain lengths [20••]. Such an interaction is predicted to destabilise folding by up to 1.5 kcal mol^{-1} (Figure 1g), which does not appear to account for the entire thermodynamic offset observed. However, when the sequence of the bound segment was mutated to reduce the extent of interaction, quantitative agreement was found between changes in the strength of this interaction and changes in the observed nascent chain stability [20••]. Ultimately, by measuring both nascent chain stability, and interactions of folded and unfolded states, these studies have provided the first direct evidence of a competition between co-translational folding and ribosome interactions.

Conclusions
This article has discussed recent progress towards a quantitative, physical chemical understanding of the emergence of protein stability during translation. However, at this stage basic questions still remain unanswered. It is now clear that some nascent chains can interact strongly with the ribosome surface as they emerge during translation, and that these interactions can perturb the thermodynamics (and kinetics) of folding, leading to an intrinsic, short-range holdase activity of the ribosome itself. However, interactions may not account for all of the energetic effects observed, which may be influenced by additional factors such as the shape of the ribosome exit tunnel [24•], weakening of the hydrophobic effect due to solvent structuring near the ribosome surface [64•], or mechanical force generated by the co-translational folding process itself [60]. The extent to which interactions may be *functional* also remains unknown. Sequestration of interacting segments may allow co-translational folding to be delayed until additional residues have been synthesised, perhaps helping to reduce the risk of co-translational misfolding [4•,34•,52•]. The basic determinants of interactions, positively charged or aromatic residues (Figure 2f), are also remarkably similar to those recognised by downstream chaperones such as trigger factor, SecB and DnaK [65–67], and so we speculate that

interactions might help regulate the engagement of emerging nascent chains with dedicated chaperones and processing factors. Fundamentally, as outlined here, the past few years have witnessed a surge of experimental studies aiming to develop quantitative measures of nascent chain stability and interactions. As such methods become established, we are confident that the next few years will see a blossoming in our basic understanding of this most fundamental cellular process.

Conflict of interest statement
Nothing declared.

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- • of special interest
- •• of outstanding interest

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