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CHAPTER TWENTY-FOUR

# Measuring Cotranslational Folding of Nascent Polypeptide Chains on Ribosomes

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## Abstract

Protein folding has been studied extensively *in vitro*, but much less is known about how folding proceeds *in vivo*. A particular distinction of folding *in vivo* is that folding begins while the nascent polypeptide chain is still undergoing synthesis by the ribosome. Studies of cotranslational protein folding are inherently much more complex than classical *in vitro* protein folding studies, and historically there have been few methods available to produce the quantities of

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pure material required for biophysical studies of the nascent chain, or assays to specifically interrogate its conformation. However, the past few years have produced dramatic methodological advances, which now place cotranslational folding studies within reach of more biochemists, enabling a detailed comparison of the earliest stages of protein folding on the ribosome to the wealth of information available for the refolding of full-length polypeptide chains *in vitro*.

# 1. Introduction

In the cell, proteins are synthesized by the ribosome as linear strings of amino acid residues (Fig. 24.1). For most proteins, this linear chain of amino acid residues must fold into a unique three-dimensional structure; correct folding is a prerequisite for proper protein function. Historically, protein folding has been studied *in vitro*, by diluting full-length polypeptide chains out of a chemical denaturant, and observing the folding process (typically via optical or NMR spectroscopic methods). This homogeneous protein solution, which unfolds and refolds reversibly, permits a detailed biophysical investigation of the thermodynamics, kinetics, conformations, and dynamics of the folding process, as well as the competing off-pathway misfolding and aggregation process. Such studies have contributed greatly to our understanding of proteins.

However, equilibrium thermodynamics can only be applied to proteins that unfold and refold reversibly, and it is often impossible to find conditions under which to study folding/unfolding of a particular protein of interest at equilibrium. A common result is that, upon dilution from chemical denaturant, the polypeptide chain will misfold and aggregate, rather than refold

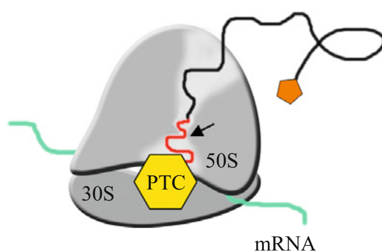


**Figure 24.1** The ribosome nascent chain (RNC) complex. Illustration of translating bacterial ribosome with a stalled nascent chain tethered at the peptidyl transferase center (PTC, hexagon). The arrow points to the narrowest part of the ribosomal tunnel, where direct interactions with the nascent stall sequence have been identified (see text). Nascent chain is drawn in black and grey (indicating the polypeptide sequence used to initiate stalling); affinity tag for purification or labeling (optional; pentagon).

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to its native structure; the chemical denaturation of GFP is a classic example of this phenomenon (Tsien, 1998). Yet, these same polypeptide chains are synthesized and fold correctly every day, often at high levels, in the crowded environment of the cell.

Early observations of this contrast between the efficiency of protein folding *in vivo* versus inefficient refolding *in vitro* focused attention on what is lost upon removing a protein from its cellular environment. These observations spurred the discovery of molecular chaperones (Hemmingsen *et al.*, 1988), and studies of how these "helper proteins" can suppress aggregation and in some cases actively promote folding to the native structure (Bukau *et al.*, 2006; Hartl and Hayer-Hartl, 2009). Nevertheless, careful proteomics studies have shown that, under normal growth conditions, the majority of proteins do not require an interaction with molecular chaperones in order to fold to the native structure (Hartl and Hayer-Hartl, 2002), casting light onto other fundamental differences between folding *in vivo* and refolding of proteins *in vitro* upon dilution from chemical denaturants.

*In vivo*, every protein enters the cellular environment vectorially, either entering the cell cytoplasm during synthesis by the ribosome or another cellular compartment upon secretion through a protein pore in a lipid bilayer. There is a profound difference between this vectorial initiation of folding *in vivo* and the refolding of a full-length protein *in vitro*. For example, *in vivo*, N-terminal segments of the polypeptide chain are the first to appear outside the ribosome exit tunnel (Fig. 24.1). These N-terminal segments can start to build interactions with nearby segments of the nascent ribosome-bound polypeptide chain, but are unable to interact with C-terminal segments of the polypeptide chain, which have not yet been synthesized or remain sequestered within the ribosome exit tunnel. One can envision a scenario where parsing out interactions between neighboring parts of the polypeptide chain could reduce the topological frustration experienced by a protein that starts to build all interactions simultaneously upon dilution of a full-length polypeptide chain out of a chemical denaturant (Clark, 2004).

Hence, while numerous studies on diverse proteins have confirmed Anfinsen's theory that proteins fold to their global energy minimum conformation, the route taken to reach that conformation can differ dramatically, depending on whether a polypeptide chain is folding cotranslationally during synthesis by the ribosome *in vivo*, or sifting through myriad possible interactions between all portions of the polypeptide chain simultaneously *in vitro*. The distinctions between cotranslational folding *in vivo* and refolding *in vitro* have been discussed in theory (Clark, 2004; Evans *et al.*, 2005a), explored computationally (Elcock, 2006; Hsiao-Mei and Jie, 2008; Wang and Klimov, 2008), and confirmed experimentally (Evans *et al.*, 2008; Fedorov and Baldwin, 1999; Frydman *et al.*, 1999). Cotranslational folding can indeed influence the folding pathway, often with profound effects on folding efficiency (Evans *et al.*, 2008; Ugrinov and Clark, submitted).

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Yet despite the potentially profound effect of cotranslational folding on protein folding mechanisms, cotranslational folding has been studied for only a small handful of proteins, and much remains unknown. This is mostly due to the experimental complexities that must be dealt with in order to study cotranslational folding. The experimental sample is no longer a pure, homogeneous polypeptide chain, but a complex mixture including nascent chains of various lengths at different points during synthesis, ribosomes, and potentially other components (molecular chaperones, the signal recognition particle (SRP), etc.). There are potentially coupled kinetics between nascent chain elongation and chain folding. There are very few experimental methods that can specifically report on the behavior of the nascent chain in such a mixture—although this is changing, and detailed below. Yet despite this complexity, great strides have been made over the past 5 years, making cotranslational measurements of nascent chain conformation increasingly accessible. Here, we discuss current state-of-the-art methods for measuring the cotranslational folding of nascent polypeptide chains. Because of the layered kinetic issue described above, these methods rely heavily on strategies that stall translation, producing a static ribosome: nascent chain complex (see below). Moreover, although this chapter describes results from cotranslational folding studies in both prokaryotic and eukaryotic systems, the detailed methods outlined below focus on cotranslational folding studies on ribosomes derived from *Escherichia coli* cells, and/or translation mixtures.

## 2. Translation and the Ribosome:Nascent Chain (RNC) Complex

Ribosomes are extremely large ribonucleoproteins, ranging in size from 2 MDa (*E. coli*) to 4 MDa (mammals). *E. coli* ribosomes consist of three separate RNA particles (rRNAs) and >50 proteins, and can be divided into two subunits: a small subunit (30 S) that binds mRNA, and a large subunit (50 S) that forms the tRNA binding sites. These two subunits dock together to produce the functional 70 S ribosome, a roughly spherical complex with the peptidyltransferase center (PTC) active site located near its center. As peptide bonds are formed at the PTC and the nascent chain elongates, it moves through a narrow exit tunnel that spans the width of the large subunit.

For the *E. coli* ribosome, the exit tunnel is approximately 100 Å long and 10–20 Å wide (Schuwirth *et al.*, 2005). Overall, the tunnel is narrowest near the PTC and grows wider as it reaches the outer surface of the large subunit, although a significant constriction occurs approximately 1/3 of the distance between the PTC and the exterior of the ribosome. The narrowness of the

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exit tunnel places significant restrictions on the extent of nascent chain folding that can occur while the nascent chain resides within the tunnel. While there appears to be enough room for chain compaction into an $\alpha$-helical conformation (Ban *et al.*, 2000; Woolhead *et al.*, 2004), there does not appear to be enough room for the nascent chain to fold back on itself to form higher-order secondary structure (such as a $\beta$-hairpin) or tertiary structure within the tunnel, except perhaps in the broader region immediately adjacent to the ribosome surface (Amit *et al.*, 2005; Lu and Deutsch, 2005a,b). This was confirmed experimentally in a classic study showing that the ribosome exit tunnel protects only the most C-terminal 30–40 aa from proteolytic digestion (Malkin and Rich, 1967).

The surface of the exit tunnel is lined with ribosomal RNA and, to a lesser extent, ribosomal proteins. For decades, the exit tunnel was considered to be a "Teflon tube" (Nissen *et al.*, 2000), devoid of interactions with the nascent polypeptide chain, as it was expected that such interactions would interfere with the smooth progress of protein synthesis and the appearance of the nascent chain. More recently, however, a few amino acid sequences have been identified that interact specifically with both RNA and protein moieties lining the tunnel walls (Nakatogawa and Ito, 2004). These interactions between the nascent chain sequence and the ribosome exit tunnel can control nascent chain conformation (Woolhead *et al.*, 2006), and regulate the conformation of the ribosome and its functions (Rospert, 2004), including protein translation (Nakatogawa and Ito, 2004). Indeed, translational regulation by interactions between the nascent chain and the ribosome exit tunnel have been exploited to produce stalled ribosome:nascent chain (RNC) complexes for detailed biophysical studies of cotranslational folding (see below).

During translation, the C-terminus of the nascent chain is covalently tethered to the PTC center. Hence as the nascent chain grows in length, its N-terminus will exit the ribosome tunnel, but remain held in close proximity to the outer surface of the ribosome. The outer surface of the ribosome immediately adjacent to the exit tunnel is rich in ribosomal proteins (Schuwirth *et al.*, 2005), some of which serve as docking sites for additional proteins such as molecular chaperones and SRP (Buskiewicz *et al.*, 2004; Ullers *et al.*, 2006).

Polypeptide chain synthesis occurs at an average rate of approximately 20 aa/s in eubacteria, and 4–6 aa/s in eukaryotes. Translation therefore occurs much more slowly than early folding steps, such as global collapse and secondary structure formation, which can occur on the order of seconds, milliseconds, or even faster (Kubelka *et al.*, 2004; Roder and Colon, 1997). Hence, for some proteins, the rate of folding might be limited by the rate of chain elongation; these represent two potentially coupled kinetic processes. As a result, a major challenge for cotranslational folding studies is to uncouple the translation and folding processes. Typically, this is accomplished by

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arresting translation at a specific point, in order to create a homogeneous solution of ribosomes bearing nascent chains of a specific sequence and length, for detailed biophysical studies of nascent chain conformations.

A further complication is that the cellular ribosome concentration is much higher than the mRNA concentration. After translation initiation, as a ribosome moves further down the mRNA, another ribosome can initiate translation right behind the first ribosome. Hence each mRNA typically bears multiple ribosomes, each at a different point in the translation process. These poly-ribosomes (polysomes) further increase sample complexity, as stalling translation at a specific point on an mRNA sequence is likely to produce one ribosome stalled at the stall point, and several more ribosomes (bearing successively shorter nascent chains) stacked up behind it. The resulting sample is therefore not homogeneous, but instead a heterogeneous mixture of nascent chain lengths. Depending on the type of conformational or dynamics analysis performed, this heterogeneity can complicate the interpretation of results. However, there are methods available to separate individual 70 S ribosomes from polysomes and analyze the resulting nascent chain length distribution within a sample (Ugrinov and Clark, submitted).

## 3. General Approaches for Generating Stalled RNC Complexes

As described above, obtaining a RNC sample that accurately reflects the vectorial synthesis of a nascent chain, while simultaneously providing the homogeneity required for quantitative biophysical assays, is a challenging task. Another challenge is achieving sample concentrations in the range necessary for precise, quantitative biophysical assays ($\mu M$–m$M$). Ribosomes are so large (see above) that the nascent chain, the subject of our investigation, represents only a tiny fraction (typically <0.1%) of the total sample mass (Johnson, 2005). In addition, the strong background signal originating from the nucleic acid and protein component of the ribosome eliminates many common spectroscopic assays from consideration, including tryptophan fluorescence, far-UV circular dichroism, and infra-red spectroscopy. The physical properties of the ribosome require the design of conformational assays with simultaneously high sensitivity and specificity for features of the nascent chain, although sensitivity can be augmented somewhat by large-scale RNC purification strategies (see below).

Currently, two strategies are broadly used to generate RNC complexes: (i) expression of an mRNA sequence lacking a stop codon (truncated mRNA), or (ii) translational stalling induced by a portion of the translating nascent chain itself. Below we briefly describe these two strategies, and discuss

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some important considerations for the production of RNC complexes created by nascent chain-induced translational stalling for biophysical analyses.

### 3.1. Truncated mRNA-based production of RNC complexes

Historically, RNC complexes bearing nascent chains of a predetermined length and sequence were produced using an mRNA lacking a stop codon as a template for *in vitro* protein expression (Haeuptle *et al.*, 1986; Hanes and Pluckthun, 1997). Translation of an mRNA lacking a stop codon hinders the recruitment of the translation termination factors responsible for the hydrolysis of the polypeptide chain from the P-site tRNA (Youngman *et al.*, 2008). As a result, a stable complex is formed between the mRNA and the ribosome bearing the synthesized nascent chain.

The elimination of the mRNA stop codon can be achieved in different ways. When an *in vitro* translation system is supplemented with a short DNA fragment complementary to a specific position of the encoding mRNA, a DNA:mRNA hybrid is formed. This double-stranded hybrid sequence is recognized by ribonuclease H (Cerritelli and Crouch, 2009; Tadokoro and Kanaya, 2009), which degrades the DNA:mRNA hybrid stretch, but not the unhybridized portions of the mRNA. This produces a truncated mRNA lacking a stop codon. Alternatively, a DNA plasmid encoding an mRNA with an early transcription terminator sequence (Hanes and Pluckthun, 1997) can be used to produce an mRNA lacking a stop codon. In either case, the mRNA lacking a stop codon is then used as the template in an *in vitro* translation system. Upon reaching the 3′ end of the mRNA, ribosomes will stall, unable to either continue synthesis or terminate translation and release the nascent chain.

While the preparation of mRNA lacking a stop codon is relatively straightforward, the use of a truncated mRNA to produce stable RNC complexes is limited to *in vitro* translation systems, which have limited translational capacity (Underwood *et al.*, 2005). *In vivo* translation of truncated mRNAs is hindered by the challenges of producing or introducing stable truncated mRNAs in live cells. Yet *in vivo*, cellular components are crowded much more closely together than they are *in vitro*, and some *in vitro* translation systems lack a full repertoire of cellular components, including components such as the secretion apparatus, which interacts with some nascent chains.

### 3.2. Nascent chain sequence-based production of RNC complexes

There are a few specific amino acid sequences that, when present in a newly synthesized polypeptide chain, can control the synthesis of downstream portions of their mRNA sequences, typically via interactions with the ribosome exit tunnel (Lovett and Rogers, 1996). Examples of such

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translational control, where the nascent chain being synthesized promotes stalling of the translating ribosome, are described in diverse organisms including viruses (Alderete *et al.*, 1999; Janzen *et al.*, 2002), eubacteria (Ambulos *et al.*, 1986; Gong and Yanofsky, 2002; Lovett, 1994), yeast (Delbecq *et al.*, 1994, 2000), fungi (Fang *et al.*, 2000, 2004), plants (Onouchi *et al.*, 2005, 2008), and higher eukaryotes (Mize and Morris, 2001; Parola and Kobilka, 1994). The mechanisms by which these nascent chains stall translation are described in detail elsewhere (Beringer, 2008; Cruz-Vera and Yanofsky, 2008; Ramu *et al.*, 2009; Yap and Bernstein, 2009). The detailed mechanisms of translational stalling, as well as the locations of the effective amino acid sequence within the nascent polypeptide chain sequence, vary considerably; nevertheless, several of these sequences contain significant amino acid similarity at key positions within the ribosome exit tunnel (Fig. 24.2). Significantly, while some nascent chain stall sequences require an additional effector molecule to trigger ribosome stalling (Fang *et al.*, 2004; Gish and Yanofsky, 1995; Werner *et al.*, 1987), other nascent chains alone are sufficient to arrest translation (Nakatogawa and Ito, 2001; Onouchi *et al.*, 2005). Most translation stall sequences are encoded in short leader peptides or upstream open reading frames (uORFs)

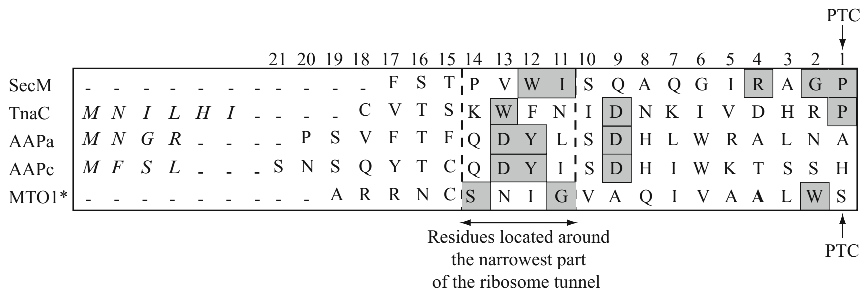


**Figure 24.2** Stalling sequences with potential uses for the production of stalled RNCs. Nascent chain amino acid residues are numbered according to their distance from the peptidyl transferase center (PTC, the point of ribosome stalling). The arrows pointing at position 1 mark the residue located at the PTC. The key residues for each stalling sequence are shown in gray boxes. The N-terminal residues of TnaC (the leader peptide from the tryptophanase operon), AAPa (arginine attenuator peptide from Neurospora crassa carbamoyl-phosphate synthetase gene arg2), and AAPc (arginine attenuator peptide from Saccharomyces cerevisiae carbamoyl-phosphate synthetase gene cpa1) peptides, which are not conserved and/or do not affect stalling, are shown in italics (Cruz-Vera and Yanofsky, 2008; Delbecq *et al.*, 2000; Fang *et al.*, 2000; Gong and Yanofsky, 2002; Hood *et al.*, 2007). The MTO1 stalling sequence from cystathionine $\gamma$-synthase (marked with an asterisk) includes the sequence RRNCSNIGVAQ, which is highly conserved in multicellular plants; residue Ser94 is located at the PTC (Ominato *et al.*, 2002).

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(Lovett and Rogers, 1996; Morris and Geballe, 2000), yet some are found in full-length protein-encoding ORFs (Nakatogawa and Ito, 2001; Ominato *et al*., 2002).

The bacterial secretion monitor protein SecM (Nakatogawa and Ito, 2001; Sarker *et al*., 2000) is a 170-aa protein that contains a 17-aa sequence near its C-terminus that is capable of stalling translation when present in the ribosome tunnel, with no requirement for an exogenous effector molecule (Nakatogawa and Ito, 2002) (Fig. 24.2). This SecM stall sequence has been exploited to stall the translation of other, diverse polypeptide chains (Evans *et al*., 2005b).

The SecM stall sequence stalls translation while lodged in the ribosomal tunnel, which can accommodate the C-terminal 30–40 aa of the nascent chain, depending on the conformation of the polypeptide chain (Ban *et al*., 2000; Gilbert *et al*., 2004; Malkin and Rich, 1967). Hence, the 17 aa of the SecM stall sequence spans only one half of the tunnel, closest to the PTC, and presumably has little effect on the conformation and dynamics of the nascent chain on the ribosome surface. The major advantage of the SecM-based translation stalling method over classical truncated mRNA methods is the capacity for *in vivo* production of stable RNC complexes (Evans *et al*., 2005b). *In vivo* assembly of SecM-stalled RNC complexes, each bearing a nascent chain of defined length and amino acid composition, provides the unique opportunity to mimic the vectorial synthesis of polypeptides by the ribosomal machinery in the context of the native cellular environment. The macromolecular crowding experienced by nascent chains *in vivo* might have a significant impact on the thermodynamic and/or kinetic properties of nascent chains, as crowding has been shown to affect macromolecular diffusion and protein folding/aggregation properties (Ellis, 2001; Ellis and Minton, 2006).

To date, SecM-mediated translation stalling has been used to produce RNC complexes bearing diverse nascent chains (Evans *et al*., 2005b; Rutkowska *et al*., 2009; Schaffitzel and Ban, 2007) (Table 24.1). Both polysome and single 70 S RNC complexes are produced, without affecting the overall cellular distribution of ribosomes and polysomes (Brandt *et al*., 2009; Ugrinov and Clark, submitted). Although prolonged stalling could result in declined ribosome recycling and a consequent decrease of cellular translation efficiency (Nakatogawa and Ito, 2001), no significant reduction in cell viability has been reported so far during *in vivo* production of stalled RNC complexes (Contreras-Martinez and DeLisa, 2007; Evans *et al*., 2005b; Schaffitzel and Ban, 2007). It appears that the SecM stall sequence can arrest the ongoing translation of virtually any polypeptide chain, underlying the potential of the method for future biophysical studies on ribosome-bound nascent chains.

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**Table 24.1** Polypeptides nascent chains created as a SecM stall sequence fusion proteins

| Nascent chain | Origin | Length(s) (aa)[a] | Translation | References |
|---|---|---|---|---|
| P22 tailspike | Bacterial phage | 248–685 | *In vivo/in vitro* | Evans *et al.* ( 2005b, 2008) |
| GFP | Eukaryotes | 89–303 | *In vivo* | Evans *et al.* (2005b), Ugrinov and Clark (submitted) |
| GFP | Eukaryotes | 373 | *In vitro* | Uemura *et al.* (2008) |
| cSNC | Synthetic | 39 | *In vivo* | Unpublished[c] |
| cAMP receptor | Eubacteria | 197[b]–209 | *In vivo* | Sunohara *et al.* (2004) |
| Spectrin | Eukaryotes | 103–367 | *In vivo/in vitro* | Hoffmann *et al.* (2006), Merz *et al.* (2008), Rutkowska *et al.* (2008) |
| FtsQ | Eubacteria | 121, 142 | *In vivo/in vitro* | Mitra *et al.* (2005), Schaffitzel and Ban (2007), Schaffitzel *et al.* (2006) |
| ICDH | Eubacteria | 35–400 | *In vivo/in vitro* | Hoffmann *et al.* (2006), Merz *et al.* (2008), Rutkowska *et al.* (2008) |
| RpoB | Eubacteria | 106–596 | *In vivo* | Rutkowska *et al.* (2008, 2009) |
| Barnase | Eubacteria | 177–236 | *In vivo* | Rutkowska *et al.* (2008, 2009) |
| MetK | Eubacteria | 112–268 | *In vivo* | Rutkowska *et al.* (2008) |
| Firefly luciferase | Eukaryotes | 113–576 | *In vivo* | Brandt *et al.* (2009), Rutkowska *et al.* (2008) |
| Anti $\beta$-gal scFv | Eubacteria | 308 | *In vivo* | Contreras-Martinez and DeLisa (2007) |
| Anti HEL scFv | Eukaryotes | 346 | *In vitro* | Ohashi *et al.* (2007) |
| Dihydrofolate reductase | Eubacteria | 284 | *In vitro* | Ohashi *et al.* (2007) |
| Yap65 | Eukaryotes | 173 | *In vitro* | Matsuura *et al.* (2007) |
| Protein D | Eubacteria | 149 | *In vitro* | Takahashi *et al.* (2009) |

[a] The length of each polypeptide is calculated according to the information provided in the listed reference(s), and references therein. The calculations include the length of the polypeptide, the SecM stall sequence, a ribosome tunnel linker (if used), and an affinity tag (if used). When more than two polypeptide chains of the same protein are used, only the lengths of the shortest and longest are shown. A small variation in the calculated size is not excluded.

[b] Shorter SecM stall sequence was used.

[c] Ugrinov and Clark (unpublished results).

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### 3.3. Caveats and limitations of stalled RNC complexes

A caveat of all ribosome stalling procedures is that the ribosome has stalled, an unnatural condition that does not occur under normal translation conditions. Hence it is quite possible that the nascent chain attached to a stalled ribosome might reach a conformation that would be kinetically inaccessible to a nascent chain on a ribosome that continues to undergo translation of downstream portions of the polypeptide chain. Technically, we are not yet able to simultaneously observe translation and cotranslational folding, although steps are being made in this direction (see Section 7). In the meantime, it is important to remember that a large amount of *in vitro* refolding experimental results collected for equilibrium conformations have assisted with descriptions of *bone fide in vitro* kinetic intermediates. And, care can be taken to design ribosome stall points to mimic endogenous local pauses in translation, resulting from rare codons (Clarke and Clark, 2008) or other effects.

The yield of stalled RNC complexes produced *in vivo* can be reduced by the action of the cellular *trans*-translation (tmRNA, SsrA) mechanism (Withey and Friedman, 2003). The *trans*-translation apparatus clears stalled bacterial ribosomes by disassembling the stalled ribosome from the engaged mRNA. The incomplete nascent chain is tagged with a specific C-terminal sequence, and directed for degradation (Keiler *et al.*, 1996; Withey and Friedman, 1999). To avoid the negative effects of *trans*-translation on SecM-mediated stalling, Δ*ssrA* bacterial strains can be used (Hallier *et al.*, 2004; Komine *et al.*, 1994). Alternatively, when an *in vitro* translation system is used, *trans*-translation can be reduced by supplementing the translation system with an anti-*ssrA* oligonucleotide (Evans *et al.*, 2005b; Schaffitzel and Ban, 2007).

It remains to be determined whether the SecM stalling procedure can be applied to translation systems from organisms other than *E. coli*, particularly eukaryotic translation systems, given the significant difference in architecture between prokaryotic and eukaryotic ribosomes (Morgan *et al.*, 2000). Possible limitations can also arise from the length of the nascent chain that can be stalled on the translating ribosome. Studies performed with P22 tailspike showed that stalling efficiency can decrease with increasing nascent chain length (Evans *et al.*, 2005b). A possible explanation is the viscous drag of a long nascent chain mostly outside the ribosome exit tunnel. In the case of tailspike, the lowest stalling efficiency was determined for a >800 aa nascent chain sequence, >4 times longer than the wild type SecM protein (170 aa).

## 4. Methods for Preparing RNC Complexes

The detailed protocol described below is based on the experimental work of our laboratory (Evans *et al.*, 2005b; Ugrinov and Clark, submitted), but the procedure is easily adjusted depending on the specific experimental

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goals and available laboratory equipment. At the end of the procedure, we provide additional comments and suggestions to refine the procedure for specific experimental goals.

## 4.1. Preliminary considerations

This procedure relies on the ribosome stalling properties of the SecM stall sequence (Evans *et al.*, 2005b; Nakatogawa and Ito, 2001). As a prerequisite, it requires an IPTG-inducible DNA expression plasmid bearing the gene for the nascent chain of interest, followed immediately by the SecM stall sequence. See Evans *et al.* (2005b) for a detailed description of such a plasmid.

## 4.2. Procedure

1. One liter of sterile Luria–Bertani (LB) broth supplemented with 100 $\mu$g/ml ampicillin is inoculated with 20 ml of an overnight stationary phase cell culture of *E. coli* transformed with the plasmid of interest. (The preparation scale can be increased or decreased, as needed. The numerous examples of generated RNC complexes (Table 24.1) demonstrate that the SecM-based methodology is applicable for practically any *E. coli*-based protein expression system.)
2. The cells are grown at 37 °C until reaching an optical density of 0.4–0.5 at 600 nm (approximately 3.5 h).
3. Protein expression is induced for 30 min by addition of IPTG to a final concentration of 0.5 m*M*.
   (There is the potential for cells expressing the SecM stall sequence for prolonged times to experience growth defects (Nakatogawa and Ito, 2004); hence expression time should be kept to a minimum.)
4. Protein expression is halted by chilling the cell culture on ice for ~10 min. All further steps are performed at 4 °C.
5. Cells are harvested by centrifugation and the cells are resuspended gently in 1 ml (for each 500 ml of cell culture) cold R buffer (50 m*M* Tris, pH 7.5; 10 m*M* $MgCl_2$; 150 m*M* KCl).
   (Complete cell resuspension produces more efficient cell lysis and higher RNC yields).
6. The resuspended cells are transferred to 2 ml microcentrifuge tubes and frozen at −80 °C for at least 30 min.
7. The frozen cells are thawed at room temperature and treated with lysozyme for 30 min. The final concentration of lysozyme is 1 mg/ml.
8. The cells are frozen again for ≥30 min at −80 °C.
9. The lysed cells are thawed at room temperature; the sample is supplemented with 50 m*M* $MgSO_4$, and treated with 100 units of DNase I (RNase free) for 30 min.

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10. The cell lysate is spun at 14,000 rpm until a solid pellet is formed (40–50 min).
11. The sample supernatant is removed, layered onto a 35% sucrose solution prepared in R buffer, and spun at 229,600×*g* for 40 min (Beckmann 70.1 Ti rotor).
12. The supernatant and the sucrose are removed; the pellet consisting of RNC complexes is gently washed and resuspended in R buffer on a rotary shaker.
    (Mechanical disruption of the pellet by the pipette tip should be avoided).
13. Calculations of ribosome concentration and nascent chain stalling efficiency are described by Evans *et al.* (2005b).

### 4.3. Additional considerations

A sample purified according the protocol above will consist of RNC complexes and ribosome-associated cellular components. Hence, this sample will permit measurements of nascent chain conformations under conditions that closely resemble the nascent chain conformations and interactions encountered *in vivo*. If further purification of the RNC complexes is required, such as removal of nonspecifically bound cellular components, removal of a labeling reagent, or separation of polysome and monosome (70 S) complexes for detailed quantitative measurements, the RNC complexes can be further refined. Separation of an RNC sample using standard size exclusion chromatography is an efficient way to achieve such separations (Woolhead *et al.*, 2004). Affinity-based chromatography methods are also suitable; however, they require specifically designed nascent chain constructs (Rutkowska *et al.*, 2009; Schaffitzel and Ban, 2007), and care must be taken that neither the affinity tag nor the purification protocol disturbs the nascent chain conformation and/or its interactions.

## 5. Biophysical Studies with RNC Complexes

The ability to produce large quantities of stalled RNC complexes has opened up a new world of experimental possibilities, expanding studies of protein conformation and dynamics in directions that were previously inaccessible. As mentioned above, the fundamental biophysical principles of protein chemistry have been derived mainly from refolding experiments using simplified experimental systems. Now, these fundamental principles can be readdressed and reevaluated from the perspective of ribosome-bound nascent chains, the true starting point for folding *in vivo*. In addition, although beyond the scope of this chapter, RNC complexes provide an

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excellent system for examining the complex interplay of interactions between nascent chains and other components of the cell, such as molecular chaperones and the secretory apparatus. Here, we review recent studies of nascent polypeptide chain folding and dynamics. These studies are grouped into broad categories, but it should be noted that some studies span multiple categories, underscoring the complexity of RNC complexes.

## 5.1. Cotranslational folding studies

The process of cotranslational folding entails acquisition of secondary and/or tertiary structures by the ribosome-bound nascent polypeptide chain prior to its complete synthesis by the ribosome (Evans *et al.,* 2005a,b; Fedorov and Baldwin, 1997; Hardesty *et al.*, 1999; Kolb *et al.*, 1995; Komar, 2009; Lim and Spirin, 1986; Ugrinov and Clark, submitted). There are now many examples of proteins that start to fold cotranslationally, supporting the important role of the vectorial appearance of the polypeptide at the surface of the ribosome, and the role of the synthesizing ribosome itself in correct folding (Clark and King, 2001; Das *et al.*, 1996; Evans *et al.*, 2008; Fedorov and Baldwin, 1997; Frydman *et al.*, 1999; Kudlicki *et al.*, 1997; Singh and Rao, 2002; Svetlov *et al.*, 2006, 2007; Ugrinov and Clark, submitted). As mentioned above, during cotranslational folding, the growing nascent chain experiences significant conformational constraint arising from the walls of the ribosomal tunnel, and numerous macromolecules gating the exit of the tunnel (Kramer *et al.*, 2009; Nissen *et al.*, 2000). In addition, the C-terminus of the nascent chain is immobilized within the bulky ribosome (Svetlov *et al.*, 2007). As a result, the folding landscape for a nascent chain is significantly different from that of a full-length polypeptide chain refolding in a test tube (Clark, 2004; Evans *et al.,* 2005a). Cotranslational folding affects the entropic and enthalpic components of the free energy for folding. Entropically, the accessible conformations of a ribosome-bound nascent chain are restricted versus the conformations of a corresponding free chain. Enthalpically, completion of the network of noncovalent bonds that stabilizes the native structure of the protein is regulated by the vectorial synthesis and appearance of the nascent chain.

The effect of the ribosome tunnel on the conformation of a nascent chain was addressed by Deutsch and coworkers, who experimentally measured the compactness of nascent chains of different lengths inside the ribosome exit tunnel (Kosolapov and Deutsch, 2009; Lu and Deutsch, 2005a,b). The experimental data was used to calculate the free energies of folding and unfolding of the nascent chain in different regions of the ribosome tunnel (Lu and Deutsch, 2005a,b). Based on their analyses, the authors propose that specific zones inside the ribosome exit tunnel play an active role inducing early nascent chain compaction and folding.

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In particular, these authors identified the distal portion of the ribosome tunnel, closest to the ribosome surface, as an ''entropic window'' where N-terminal portions of a nascent chain can explore conformational space (Kosolapov and Deutsch, 2009).

Johnson and coworkers have developed techniques for Förster resonance energy transfer (FRET)-based measurements that have revolutionized the field of cotranslational folding (Johnson, 2005; Woolhead *et al.*, 2004, 2006). FRET provides information on the distance between two fluorescent probes, and so allows evaluation of the conformational ensembles populated by a doubly labeled polypeptide chain in the process of folding/unfolding (Haas, 2005; Stryer, 1978). When the measurements are performed with a series of homogeneous ribosome: nascent chain complexes having specifically labeled nascent chains with a predetermined length, information for the folding of the nascent chain at each step of the biosynthetic process can be gained (Woolhead *et al.*, 2004, 2006). For example, Johnson and coworkers used FRET measurements to determine that transmembrane helical segments of the nascent chain adopt a helical conformation deep within the ribosome exit tunnel, and the ribosome tunnel environment is necessary for formation of this conformation (Woolhead *et al.*, 2004).

Diverse cotranslational folding studies with purified RNC complexes have also revealed the presence of foldable, on-pathway nascent chain conformations on the surface of the ribosome (Clark and King, 2001; Evans *et al.*, 2008; Frydman *et al.*, 1994; Kudlicki *et al.*, 1994, 1995; Ugrinov and Clark, submitted). Studies performed with firefly luciferase and P22 tailspike protein have demonstrated that the conformations of these nascent chains on the surface of the ribosome are distinct from the conformations populated by the protein upon dilution out of a chemical denaturant *in vitro* (Clark and King, 2001; Evans *et al.*, 2008; Frydman *et al.*, 1999). Similarly, for full-length GFP nascent chains, cotranslational conformations have been shown to lead to a much higher folding yield than the conformations populated by full-length free GFP chains unfolded in a chemical denaturant (Ugrinov and Clark, submitted).

## 5.2. Studies of nascent chain dynamics

Very little is known about the conformational flexibility of the newly synthesized peptide inside the ribosome tunnel, or on the surface of the ribosome. Nascent chain dynamics directly reflect the rigidity of the molecule as well as the conformational space accessible for the growing polypeptide chain. Recently, however, NMR and fluorescence spectroscopy have made possible direct measurements of the dynamics of various nascent chains (Ellis *et al.*, 2008; Hsu *et al.*, 2007, 2009), and highlight the possibility for future advanced thermodynamic and kinetic studies of RNC complexes. Results derived from both NMR relaxation measurements and time

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resolved fluorescence measurements can be used for evaluation of the conformational entropy of the experimental system and changes in the heat capacity during protein folding and unfolding (Yang and Kay, 1996; Yang *et al.*, 1997).

## 5.3. Complementary approaches to cotranslational folding

*In vitro* protein folding studies with truncated free polypeptide chains (no ribosomes present), as well as computational cotranslational folding studies, are briefly discussed here. These studies complement the experimental results on RNC complexes described above, and provide an experimental and theoretical foundation for conformational changes occurring with increasing polypeptide chain length, with or without confinement by a ribosome surface.

A comprehensive *in vitro* structural analysis of C-terminally truncated chains of two small globular proteins, barnase and chymotrypsin inhibitor 2 (CI2), showed that significant stable native-like conformations are present only in polypeptide chains with lengths close to the size of the full-length protein (Neira and Fersht, 1999a,b). Similar results were shown for C-terminally truncated chains of two other small proteins, staphylococcal nuclease and sperm whale apomyoglobin: these polypeptide chains acquired their native state only when their C-terminal residues were present (Chow *et al.*, 2003; Flanagan *et al.*, 1992). Yet these proteins are all quite small, meaning that the C-terminal 20–40 aa of the newly synthesized chain that resides within the ribosome tunnel even at the termination of translation represents a significant portion of the polypeptide chain. Hence it would not be surprising for these four proteins to acquire stable native-like structures only after ribosome release (Chow *et al.*, 2003; Flanagan *et al.*, 1992; Neira and Fersht, 1999a,b). It is important to note, however, that the *in vitro* studies performed with these proteins have shown that even their short truncated chains can form compact structures with some long range (but perhaps non-native) interactions (Chow *et al.*, 2003; Flanagan *et al.*, 1992; Neira and Fersht, 1999a,b). Hence cotranslational formation of similar compact structures cannot be excluded. A direct comparison of the folding of free, truncated polypeptide chains with the folding of nascent chains of various lengths, tethered to the ribosome, would ensure proper understanding of the processes governing vectorial synthesis and folding of these proteins.

Numerous cotranslational folding simulation studies have emerged in recent years (Elcock, 2006; Hsiao-Mei and Jie, 2008; Huard *et al.*, 2006; Lydia *et al.*, 2006; Peiyu and Dmitri, 2008). Comprehensive molecular simulations of the cotranslational folding of CI2, barnase, and Semliki forest virus protein (SFVP) were presented by Elcock (2006). For the purposes of the simulations, the conformations adopted by the polypeptide chains were represented in terms of fraction of native-like contacts between the amino

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acid residues, and the changes in the energy of folding of the "nascent" polypeptides were monitored as a function of the native-like contacts. Importantly, the values for the folded and unfolded state energies, as well as the acquisition of native-like contacts, were standardized to experimentally calculated values (Neira and Fersht, 1999a,b). These simulations supported the results of the C-terminal truncations described above, suggesting that CI2 and barnase are unlikely to fold to a stable, native-like structure cotranslationally. In contrast, cotranslational folding was observed for the N-terminal domain of the larger multidomain protein SFVP, which is known to fold cotranslationally into an active protease structure (Nicola *et al.*, 1999).

Lattice-based simulations have been used to measure kinetics for cotranslational folding versus refolding of small single domain proteins (Peiyu and Dmitri, 2008). The simulations showed slower, more complex kinetics for "cotranslational" folding, compared to the folding under "*in vitro*" conditions. The different folding mechanisms were attributed to different conformations populated by the free chains and the ribosome-bound chains at the beginning of the reaction and as folding progressed (Peiyu and Dmitri, 2008). Similar observations were reported for HP (hydrophobic/polar) lattice simulation, where the growing nascent chain populated ground states different from the global energy minimum state (Huard *et al.*, 2006).

# 6. Measuring Nascent Chain Cotranslational Folding and Rigidity by Limited Protease Digestion

Resistance of a protein to a limited protease digestion is commonly used to investigate the compactness of a polypeptide chain (Hubbard, 1998; Park and Marqusee, 2004). The method relies on the higher susceptibility of unstructured and extended conformations to protease cleavage; in contrast, rigid (and hence folded) regions are protected from digestion. Of course, it is also possible that the ribosome itself might provide a steric blockade that shields the nascent chain from the protease. Nevertheless, carefully controlled experiments with diverse RNC complexes have shown that limited protease digestion can provide crucial information on the cotranslational conformations of nascent proteins (Evans *et al.*, 2008; Frydman *et al.*, 1999; Malkin and Rich, 1967; Picking *et al.*, 1992).

## 6.1. Preliminary considerations

Digestion conditions must be chosen that do not affect the integrity of the ribosome itself. In general, digestion times should be kept as short as possible, to minimize digestion-time dependent changes in the nascent

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chain conformation. When a nonspecific protease such as proteinase K is used for the limited digest, this method is generally applicable to all RNC complexes. Moreover, the identification of resulting digestion fragments will provide unbiased information on nascent chain compaction, rigidity, and folding (and potentially, ribosome shielding). Alternatively, a specific protease cleavage site (recognized by Factor Xa, enterokinase, TEV protease, etc.) could be introduced at a desired position in the ribosome-bound nascent chain. Cleavage at this position would indicate that the protease cleavage site resides at an unstructured or otherwise accessible location on the nascent chain conformation. The protocol below should be considered as a guideline rather than a detailed procedure, since it is based on the specific aims of our laboratory (Evans *et al.*, 2008).

### 6.2. Procedure

1. Fresh RNC complexes are purified in R buffer as described above. (Any freezing/thawing prior to the treatment with proteinase K should be avoided. The proteinase K reaction is performed at 4 °C.)
2. Approximately 1 ml of RNC complexes are mixed with an appropriate volume of proteinase K stock solution (prepared in R buffer) to a final volume of 1 ml and final concentration of proteinase K of 1 $\mu$g/ml. (The concentration of the RNC complexes, and the [RNC]/[proteinase K] ratio, will vary and is determined by the properties of the specific nascent chain and the goals of the experiment. A final concentration of 1 $\mu$g/ml proteinase K and digestion times of <20 min will keep ribosomes intact throughout the experiment.)
3. At various digestion time points, proteinase K is inhibited by addition of 1 m*M* PMSF (phenylmethylsulphonyl fluoride, a serine protease inhibitor) to the reaction mixture.
4. The sample is incubated with PMSF for 15 min and layered onto a 35% sucrose solution prepared in R buffer.
5. The sample is spun at 229,600$\times g$ for 40 min (Beckmann 70.1 Ti rotor), and the ribosome-free supernatant is collected.
6. The supernatant, which contains the protease resistant fragments, is analyzed by gel electrophoresis, immunoblotting, mass spectrometry, etc. (e.g., see (Evans *et al.*, 2008)).

## 7. Future Directions

While cotranslational folding studies have made great strides recently, much work remains. For example, an important bottleneck is the current lack of methods suitable to measure cotranslational folding while nascent

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chain synthesis is underway. An experiment of this sort would remove the caveat that the conformations populated on a stalled ribosome might not resemble the conformations populated if translation were allowed to continue uninterrupted. Recent advances in single molecule techniques to monitor protein translation (Blanchard, 2009; Katranidis *et al*., 2009; Uemura *et al*., 2008) hold promise for future technical advances in this direction.

Finally, many of the considerations described here for vectorial, cotranslational folding of nascent polypeptide chains on the ribosome also apply to vectorial, cosecretory folding of polypeptide chains as they are moved across a lipid bilayer from one cellular compartment to another. While landmark studies have provided some information on the direction of chain transport across membranes (Junker *et al*., 2009), and the effect of premature folding on secretion efficiency (Teschke *et al*., 1991), the conformations of these polypeptides as they appear in their destination compartment, and the influence of these conformations on final folding yield, are much less well understood.

## *REFERENCES*


Alderete, J. P., *et al.* (1999). Translational effects of mutations and polymorphisms in a repressive upstream open reading frame of the human cytomegalovirus UL4 gene. *J. Virol.* **73,** 8330–8337.

Ambulos, N. P. Jr., *et al.* (1986). Analysis of the regulatory sequences needed for induction of the chloramphenicol acetyltransferase gene *cat-86* by chloramphenicol and amicetin. *J. Bacteriol.* **167,** 842–849.

Amit, M., *et al.* (2005). A crevice adjoining the ribosome tunnel: Hints for cotranslational folding. *FEBS Lett.* **579,** 3207–3213.

Ban, N., *et al.* (2000). The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science* **289,** 905–920.

Beringer, M. (2008). Modulating the activity of the peptidyl transferase center of the ribosome. *RNA* **14,** 795–801.

Blanchard, S. C. (2009). Single-molecule observations of ribosome function. *Curr. Opin. Struct. Biol.* **19,** 103–109.

Brandt, F., *et al.* (2009). The native 3D organization of bacterial polysomes. *Cell* **136,** 261–271.

Bukau, B., *et al.* (2006). Molecular chaperones and protein quality control. *Cell* **125,** 443–451.

Buskiewicz, I., *et al.* (2004). Trigger factor binds to ribosome-signal-recognition particle (SRP) complexes and is excluded by binding of the SRP receptor. *Proc. Natl. Acad. Sci. USA* **101,** 7902–7906.

Cerritelli, S. M., and Crouch, R. J. (2009). Ribonuclease H: The enzymes in eukaryotes. *FEBS J.* **276,** 1494–1505.

Chow, C. C., *et al.* (2003). Chain length dependence of apomyoglobin folding: Structural evolution from misfolded sheets to native helices. *Biochemistry* **42,** 7090–7099.

Clark, P. L. (2004). Protein folding in the cell: Reshaping the folding funnel. *Trends Biochem. Sci.* **29,** 527–534.

Author's personal copy



Clark, P. L., and King, J. (2001). A newly synthesized, ribosome-bound polypeptide chain adopts conformations dissimilar from early *in vitro* refolding intermediates. *J. Biol. Chem.* **276,** 25411–25420.

Clarke, T. F. IV., and Clark, P. L. (2008). Rare codons cluster. *PLoS ONE* **3,** e3412.

Contreras-Martinez, L. M., and DeLisa, M. P. (2007). Intracellular ribosome display via SecM translation arrest as a selection for antibodies with enhanced cytosolic stability. *J. Mol. Biol.* **372,** 513–524.

Cruz-Vera, L. R., and Yanofsky, C. (2008). Conserved residues Asp16 and Pro24 of TnaC-tRNAPro participate in tryptophan induction of Tna operon expression. *J. Bacteriol.* **190,** 4791–4797.

Das, B., *et al.* (1996). *In vitro* protein folding by ribosomes from *Escherichia coli*, wheat germ and rat liver: The role of the 50 S particle and its 23 S rRNA. *Eur. J. Biochem.* **235,** 613–621.

Delbecq, P., *et al.* (1994). A segment of mRNA encoding the leader peptide of the CPA1 gene confers repression by arginine on a heterologous yeast gene transcript. *Mol. Cell. Biol.* **14,** 2378–2390.

Delbecq, P., *et al.* (2000). Functional analysis of the leader peptide of the yeast gene CPA1 and heterologous regulation by other fungal peptides. *Curr. Genet.* **38,** 105–112.

Elcock, A. H. (2006). Molecular simulations of cotranslational protein folding: Fragment stabilities, folding cooperativity, and trapping in the ribosome. *PLoS Comput. Biol.* **2,** e98.

Ellis, R. J. (2001). Macromolecular crowding: Obvious but underappreciated. *Trends Biochem. Sci.* **26,** 597–604.

Ellis, R. J., and Minton, A. P. (2006). Protein aggregation in crowded environments. *Biol. Chem.* **387,** 485–497.

Ellis, J. P., *et al.* (2008). Chain dynamics of nascent polypeptides emerging from the ribosome. *ACS Chem. Biol.* **3,** 555–566.

Evans, M. S., *et al.* (2005a). Conformations of co-translational folding intermediates. *Protein Pept. Lett.* **12,** 189–195.

Evans, M. S., *et al.* (2005b). Homogeneous stalled ribosome nascent chain complexes produced *in vivo* or *in vitro*. *Nat. Methods* **2,** 757–762.

Evans, M. S., *et al.* (2008). Cotranslational folding promotes $\beta$-helix formation and avoids aggregation *in vivo*. *J. Mol. Biol.* **383,** 683–692.

Fang, P., *et al.* (2000). Evolutionarily conserved features of the arginine attenuator peptide provide the necessary requirements for its function in translational regulation. *J. Biol. Chem.* **275,** 26710–26719.

Fang, P., *et al.* (2004). A nascent polypeptide domain that can regulate translation elongation. *Proc. Natl. Acad. Sci. USA* **101,** 4059–4064.

Fedorov, A. N., and Baldwin, T. O. (1997). Cotranslational protein folding. *J. Biol. Chem.* **272,** 32715–32718.

Fedorov, A. N., and Baldwin, T. O. (1999). Process of biosynthetic protein folding determines the rapid formation of native structure. *J. Mol. Biol.* **294,** 579–586.

Flanagan, J. M., *et al.* (1992). Truncated staphylococcal nuclease is compact but disordered. *Proc. Natl. Acad. Sci. USA* **89,** 748–752.

Frydman, J., *et al.* (1994). Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. *Nature* **370,** 111–117.

Frydman, J., *et al.* (1999). Co-translational domain folding as the structural basis for the rapid *de novo* folding of firefly luciferase. *Nat. Struct. Biol.* **6,** 697–705.

Gilbert, R. J., *et al.* (2004). Three-dimensional structures of translating ribosomes by cryo-EM. *Mol. Cell.* **14,** 57–66.

Gish, K., and Yanofsky, C. (1995). Evidence suggesting *cis* action by the TnaC leader peptide in regulating transcription attenuation in the tryptophanase operon of *Escherichia coli*. *J. Bacteriol.* **177,** 7245–7254.

Author's personal copy



Gong, F., and Yanofsky, C. (2002). Instruction of translating ribosome by nascent peptide. *Science* **297,** 1864–1867.
Haas, E. (2005). The study of protein folding and dynamics by determination of intramolecular distance distributions and their fluctuations using ensemble and single-molecule FRET measurements. *Chemphyschem* **6,** 858–870.
Haeuptle, M. T., *et al.* (1986). Translation arrest by oligodeoxynucleotides complementary to mRNA coding sequences yields polypeptides of predetermined length. *Nucleic Acids Res.* **14,** 1427–1448.
Hallier, M., *et al.* (2004). Pre-binding of small protein B to a stalled ribosome triggers trans-translation. *J. Biol. Chem.* **279,** 25978–25985.
Hanes, J., and Pluckthun, A. (1997). *In vitro* selection and evolution of functional proteins by using ribosome display. *Proc. Natl. Acad. Sci. USA* **94,** 4937–4942.
Hardesty, B., *et al.* (1999). Co-translational folding. *Curr. Opin. Struct. Biol.* **9,** 111–114.
Hartl, F. U., and Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: From nascent chain to folded protein. *Science* **295,** 1852–1858.
Hartl, F. U., and Hayer-Hartl, M. (2009). Converging concepts of protein folding *in vitro* and *in vivo*. *Nat. Struct. Mol. Biol.* **16,** 574–581.
Hemmingsen, S. M., *et al.* (1988). Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature* **333,** 330–334.
Hoffmann, A., *et al.* (2006). Trigger factor forms a protective shield for nascent polypeptides at the ribosome. *J. Biol. Chem.* **281,** 6539–6545.
Hood, H. M., *et al.* (2007). Evolutionary changes in the fungal carbamoyl-phosphate synthetase small subunit gene and its associated upstream open reading frame. *Fungal Genet. Biol.* **44,** 93–104.
Hsiao-Mei, L., and Jie, L. (2008). A model study of protein nascent chain and cotranslational folding using hydrophobic-polar residues. *Proteins: Struct. Funct. Bioinform.* **70,** 442–449.
Hsu, S. T., *et al.* (2007). Structure and dynamics of a ribosome-bound nascent chain by NMR spectroscopy. *Proc. Natl. Acad. Sci. USA* **104,** 16516–16521.
Hsu, S. D., *et al.* (2009). Structure, Dynamics and folding of an immunoglobulin domain of the gelation factor (ABP-120) from *Dictyostelium discoideum*. *J. Mol. Biol.* **388,** 865–879.
Huard, F. P., *et al.* (2006). Modelling sequential protein folding under kinetic control. *Bioinformatics* **22,** e203–e210.
Hubbard, S. J. (1998). The structural aspects of limited proteolysis of native proteins. *Biochim. Biophys. Acta.* **1382,** 191–206.
Janzen, D. M., *et al.* (2002). Inhibition of translation termination mediated by an interaction of eukaryotic release factor 1 with a nascent peptidyl-tRNA. *Mol. Cell. Biol.* **22,** 8562–8570.
Johnson, A. E. (2005). The co-translational folding and interactions of nascent protein chains: A new approach using fluorescence resonance energy transfer. *FEBS Lett.* **579,** 916–920.
Junker, M., *et al.* (2009). Vectorial transport and folding of an autotransporter virulence protein during outer membrane secretion. *Mol. Microbiol.* **71,** 1323–1332.
Katranidis, A., *et al.* (2009). Fast biosynthesis of GFP molecules: A single-molecule fluorescence study. *Angew. Chem. Int. Ed.* **48,** 1758–1761.
Keiler, K. C., *et al.* (1996). Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. *Science* **271,** 990–993.
Kolb, V. A., *et al.* (1995). Cotranslational folding of proteins. *Biochem. Cell Biol.* **73,** 1217–1220.
Komar, A. A. (2009). A pause for thought along the co-translational folding pathway. *Trends Biochem. Sci.* **34,** 16–24.
Komine, Y., *et al.* (1994). A tRNA-like structure is present in 10Sa RNA, a small stable RNA from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **91,** 9223–9227.

Author's personal copy



Kosolapov, A., and Deutsch, C. (2009). Tertiary interactions within the ribosomal exit tunnel. *Nat. Struct. Mol. Biol.* **16,** 405–411.

Kramer, G., *et al.* (2009). The ribosome as a platform for co-translational processing, folding and targeting of newly synthesized proteins. *Nat. Struct. Mol. Biol.* **16,** 589–597.

Kubelka, J., *et al.* (2004). The protein folding 'speed limit'. *Curr. Opin. Struct. Biol.* **14,** 76–88.

Kudlicki, W., *et al.* (1994). Activation and release of enzymatically inactive, full-length rhodanese that is bound to ribosomes as peptidyl-tRNA. *J. Biol. Chem.* **269,** 16549–16553.

Kudlicki, W., *et al.* (1995). Elongation and folding of nascent ricin chains a peptidyl-tRNA on ribosomes: The effect of amino acid deletion on these processes. *J. Mol. Biol.* **252,** 203–212.

Kudlicki, W., *et al.* (1997). Ribosomes and ribosomal RNA as chaperones for folding of proteins. *Fold. Des.* **2,** 101–108.

Lim, V. I., and Spirin, A. S. (1986). Stereochemical analysis of ribosomal transpeptidation. Conformation of nascent peptide. *J. Mol. Biol.* **188,** 565–574.

Lovett, P. S. (1994). Nascent peptide regulation of translation. *J. Bacteriol.* **176,** 6415–6417.

Lovett, P. S., and Rogers, E. J. (1996). Ribosome regulation by the nascent peptide. *Microbiol. Rev.* **60,** 366–385.

Lu, J., and Deutsch, C. (2005a). Folding zones inside the ribosomal exit tunnel. *Nat. Struct. Mol. Biol.* **12,** 1123–1129.

Lu, J., and Deutsch, C. (2005b). Secondary structure formation of a transmembrane segment in Kv channels. *Biochemistry* **44,** 8230–8243.

Lydia, M. C. M., *et al.* (2006). Protein translocation through a tunnel induces changes in folding kinetics: A lattice model study. *Biotechnol. Bioeng.* **94,** 105–117.

Malkin, L. I., and Rich, A. (1967). Partial resistance of nascent polypeptide chains to proteolytic digestion due to ribosomal shielding. *J. Mol. Biol.* **26,** 329–346.

Matsuura, T., *et al.* (2007). Nascent chain, mRNA, and ribosome complexes generated by a pure translation system. *Biochem. Biophys. Res. Commun.* **352,** 372–377.

Merz, F., *et al.* (2008). Molecular mechanism and structure of trigger factor bound to the translating ribosome. *EMBO J.* **27,** 1622–1632.

Mitra, K., *et al.* (2005). Structure of the *E. coli* protein-conducting channel bound to a translating ribosome. *Nature* **438,** 318–324.

Mize, G. J., and Morris, D. R. (2001). A mammalian sequence-dependent upstream open reading frame mediates polyamine-regulated translation in yeast. *RNA* **7,** 374–381.

Morgan, D. G., *et al.* (2000). A comparison of the yeast and rabbit 80 S ribosome reveals the topology of the nascent chain exit tunnel, inter-subunit bridges and mammalian rRNA expansion segments. *J. Mol. Biol.* **301,** 301–321.

Morris, D. R., and Geballe, A. P. (2000). Upstream open reading frames as regulators of mRNA translation. *Mol. Cell. Biol.* **20,** 8635–8642.

Nakatogawa, H., and Ito, K. (2001). Secretion monitor, SecM, undergoes self-translation arrest in the cytosol. *Mol. Cell.* **7,** 185–192.

Nakatogawa, H., and Ito, K. (2002). The ribosomal exit tunnel functions as a discriminating gate. *Cell.* **108,** 629–636.

Nakatogawa, H., and Ito, K. (2004). Intraribosomal regulation of expression and fate of proteins. *ChemBioChem.* **5,** 48–51.

Neira, J. L., and Fersht, A. R. (1999a). Acquisition of native-like interactions in C-terminal fragments of barnase. *J. Mol. Biol.* **287,** 421–432.

Neira, J. L., and Fersht, A. R. (1999b). Exploring the folding funnel of a polypeptide chain by biophysical studies on protein fragments. *J. Mol. Biol.* **285,** 1309–1333.

Nicola, A. V., *et al.* (1999). Co-translational folding of an alphavirus capsid protein in the cytosol of living cells. *Nat. Cell. Biol.* **1,** 341–345.

Author's personal copy



Nissen, P., *et al.* (2000). The structural basis of ribosome activity in peptide bond synthesis. *Science* **289,** 920–930.

Ohashi, H., *et al.* (2007). Efficient protein selection based on ribosome display system with purified components. *Biochem. Biophys. Res. Commun.* **352,** 270–276.

Ominato, K., *et al.* (2002). Identification of a short highly conserved amino acid sequence as the functional region required for posttranscriptional autoregulation of the cystathionine gamma-synthase gene in *Arabidopsis*. *J. Biol. Chem.* **277,** 36380–36386.

Onouchi, H., *et al.* (2005). Nascent peptide-mediated translation elongation arrest coupled with mRNA degradation in the CGS1 gene of *Arabidopsis*. *Genes Dev.* **19,** 1799–1810.

Onouchi, H., *et al.* (2008). Nascent peptide-mediated translation elongation arrest of *Arabidopsis thaliana* CGS1 mRNA occurs autonomously. *Plant Cell Physiol.* **49,** 549–556.

Park, C., and Marqusee, S. (2004). Probing the high energy states in proteins by proteolysis. *J. Mol. Biol.* **343,** 1467–1476.

Parola, A. L., and Kobilka, B. K. (1994). The peptide product of a 5' leader cistron in the beta 2 adrenergic receptor mRNA inhibits receptor synthesis. *J. Biol. Chem.* **269,** 4497–4505.

Peiyu, W., and Dmitri, K. K. (2008). Lattice simulations of cotranslational folding of single domain proteins. *Proteins: Struct. Funct. Bioinform.* **70,** 925–937.

Picking, W. D., *et al.* (1992). Fluorescence characterization of the environment encountered by nascent polyalanine and polyserine as they exit *Escherichia coli* ribosomes during translation. *Biochemistry* **31,** 2368–2375.

Ramu, H., *et al.* (2009). Programmed drug-dependent ribosome stalling. *Mol. Microbiol.* **71,** 811–824.

Roder, H., and Colon, W. (1997). Kinetic role of early intermediates in protein folding. *Curr. Opin. Struct. Biol.* **7,** 15–28.

Rospert, S. (2004). Ribosome function: Governing the fate of a nascent polypeptide. *Curr. Biol.* **14,** R386–R388.

Rutkowska, A., *et al.* (2008). Dynamics of trigger factor interaction with translating ribosomes. *J. Biol. Chem.* **283,** 4124–4132.

Rutkowska, A., *et al.* (2009). Large-scale purification of ribosome-nascent chain complexes for biochemical and structural studies. *FEBS Lett.* **583,** 2407–2413.

Sarker, S., *et al.* (2000). Revised translation start site for secM defines an atypical signal peptide that regulates *Escherichia coli* secA expression. *J. Bacteriol.* **182,** 5592–5595.

Schaffitzel, C., and Ban, N. (2007). Generation of ribosome nascent chain complexes for structural and functional studies. *J. Struct. Biol.* **158,** 463–471.

Schaffitzel, C., *et al.* (2006). Structure of the *E. coli* signal recognition particle bound to a translating ribosome. *Nature* **444,** 503–506.

Schuwirth, B. S., *et al.* (2005). Structures of the bacterial ribosome at 3.5 Å resolution. *Science* **310,** 827–834.

Singh, R., and Rao, C. M. (2002). Chaperone-like activity and surface hydrophobicity of 70 S ribosome. *FEBS Lett.* **527,** 234–238.

Stryer, L. (1978). Fluorescence energy transfer as a spectroscopic ruler. *Annu. Rev. Biochem.* **47,** 819–846.

Sunohara, T., *et al.* (2004). Ribosome stalling during translation elongation induces cleavage of mRNA being translated in *Escherichia coli*. *J. Biol. Chem.* **279,** 15368–15375.

Svetlov, M. S., *et al.* (2006). Effective cotranslational folding of firefly luciferase without chaperones of the Hsp70 family. *Protein Sci.* **15,** 242–247.

Svetlov, M. S., *et al.* (2007). Folding of the firefly luciferase polypeptide chain with immobilized C-terminus. *Mol. Biol. (Mosk).* **41,** 96–102.

Tadokoro, T., and Kanaya, S. (2009). Ribonuclease H: Molecular diversities, substrate binding domains, and catalytic mechanism of the prokaryotic enzymes. *FEBS J.* **276,** 1482–1493.

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Takahashi, S., *et al.* (2009). Real-time monitoring of cell-free translation on a quartz-crystal microbalance. *J. Am. Chem. Soc.* **131,** 9326–9332.

Teschke, C. M., *et al.* (1991). Mutations that affect the folding of ribose-binding protein selected as suppressors of a defect in export in *Escherichia coli*. *J. Biol. Chem.* **266,** 11789–11796.

Tsien, R. Y. (1998). The green fluorescent protein. *Annu. Rev. Biochem.* **67,** 509–544.

Uemura, S., *et al.* (2008). Single-molecule imaging of full protein synthesis by immobilized ribosomes. *Nucleic Acids Res.* **36,** e70.

Ugrinov, K. G., and Clark, P. L. (submitted). Co-translational folding increases GFP folding yield.

Ullers, R. S., *et al.* (2006). Sequence-specific interactions of nascent *Escherichia coli* polypeptides with trigger factor and signal recognition particle. *J. Biol. Chem.* **281,** 13999–14005.

Underwood, K. A., *et al.* (2005). Quantitative polysome analysis identifies limitations in bacterial cell-free protein synthesis. *Biotechnol. Bioeng.* **91,** 425–435.

Wang, P., and Klimov, D. K. (2008). Lattice simulations of cotranslational folding of single domain proteins. *Proteins* **70,** 925–937.

Werner, M., *et al.* (1987). The leader peptide of yeast gene CPA1 is essential for the translational repression of its expression. *Cell* **49,** 805–813.

Withey, J., and Friedman, D. (1999). Analysis of the role of trans-translation in the requirement of tmRNA for λmmP22 growth in *Escherichia coli*. *J. Bacteriol.* **181,** 2148–2157.

Withey, J. H., and Friedman, D. I. (2003). A salvage pathway for protein structures: tmRNA and trans-translation. *Annu. Rev. Microbiol.* **57,** 101–123.

Woolhead, C. A., *et al.* (2004). Nascent membrane and secretory proteins differ in FRET-detected folding far inside the ribosome and in their exposure to ribosomal proteins. *Cell* **116,** 725–736.

Woolhead, C. A., *et al.* (2006). Translation arrest requires two-way communication between a nascent polypeptide and the ribosome. *Mol. Cell.* **22,** 587–598.

Yang, D., and Kay, L. E. (1996). Contributions to conformational entropy arising from bond vector fluctuations measured from NMR-derived order parameters: Application to protein folding. *J. Mol. Biol.* **263,** 369–382.

Yang, D., *et al.* (1997). Contributions to protein entropy and heat capacity from bond vector motions measured by NMR spin relaxation. *J. Mol. Biol.* **272,** 790–804.

Yap, M. N., and Bernstein, H. D. (2009). The plasticity of a translation arrest motif yields insights into nascent polypeptide recognition inside the ribosome tunnel. *Mol. Cell.* **34,** 201–211.

Youngman, E. M., *et al.* (2008). Peptide release on the ribosome: Mechanism and implications for translational control. *Annu. Rev. Microbiol.* **62,** 353–373.