

In vivo translation rates can substantially delay the cotranslational folding of the *Escherichia coli* cytosolic proteome

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A question of fundamental importance concerning protein folding in vivo is whether the kinetics of translation or the thermodynamics of the ribosome nascent chain (RNC) complex is the major determinant of cotranslational folding behavior. This is because translation rates can reduce the probability of cotranslational folding below that associated with arrested ribosomes, whose behavior is determined by the equilibrium thermodynamics of the RNC complex. Here, we combine a chemical kinetic equation with genomic and proteomic data to predict domain folding probabilities as a function of nascent chain length for *Escherichia coli* cytosolic proteins synthesized on both arrested and continuously translating ribosomes. Our results indicate that, at in vivo translation rates, about one-third of the *Escherichia coli* cytosolic proteins exhibit cotranslational folding, with at least one domain in each of these proteins folding into its stable native structure before the full-length protein is released from the ribosome. The majority of these cotranslational folding domains are influenced by translation kinetics which reduces their probability of cotranslational folding and consequently increases the nascent chain length at which they fold into their native structures. For about 20% of all cytosolic proteins this delay in folding can exceed the length of the completely synthesized protein, causing one or more of their domains to switch from co- to posttranslational folding solely as a result of the in vivo translation rates. These kinetic effects arise from the difference in time scales of folding and amino-acid addition, and they represent a source of metastability in *Escherichia coli*'s proteome.

systems biology | synonymous codons | chemical kinetics | chaperone | aggregation

During translation, the ribosome coordinates a series of complex processes, including the unidirectional translocation of an mRNA molecule one codon at a time, the selection for each codon of a complementary tRNA from the cytosol, and the catalysis of the peptide bond. The result is the synthesis of the corresponding nascent chain and its extrusion through a tunnel embedded within the large ribosomal subunit. As individual domains of a multidomain protein emerge from this exit tunnel, they have the opportunity to form secondary and tertiary structures and fold into their native or native-like structures while the rest of the nascent chain is still being synthesized (Fig. 1A). Such cotranslational folding has been shown to occur both in vivo and in vitro (1–3), with large multidomain proteins being more likely to exhibit cotranslational folding than small single domain proteins (4).

The time-dependent, irreversible nature of translation means that cotranslational folding occurs out of equilibrium. Consequently, the interplay of time scales arising from different processes involving the ribosome nascent chain (RNC) complex has the potential to modulate the probability that cotranslational folding occurs (5, 6). There are at least three time scales that can have an impact on the cotranslational folding process at nascent chain length i : (a) the mean time it takes to add an amino acid to the growing nascent chain (denoted $\tau_{A,i}$) (7), (b) the mean time

of domain unfolding (denoted $\tau_{U,i}$), and (c) the mean time of domain folding (denoted $\tau_{F,i}$). The cotranslational folding of the multidomain SufI protein, for example, was abolished when the translation rate ($1/\tau_{A,i}$) was increased by either replacing slow translating codons located near domain boundaries with fast translating codons or adding excess amounts of aminoacyl-tRNA (8). Moreover, a simulation study that examined the cotranslational folding of protein G (Fig. 1A) found that uniformly speeding up translation suppressed cotranslational folding and that placing slow or fast translating codons at specific points along an mRNA molecule could, respectively, increase or decrease the amount of cotranslational folding that occurred (7). Thus, increasing the translation rate can shift the cotranslational folding curve toward longer nascent chain lengths (Fig. 1B and C).

Although the cotranslational folding properties of an increasing number of proteins are being characterized experimentally in vitro (9, 10), very little is known about the importance of cotranslational folding in vivo for entire proteomes of specific organisms. Therefore, one fundamental question that arises concerns the percentage of an organism's proteome that folds cotranslationally. A second fundamental question is to what extent proteins that fold cotranslationally do so under a regime governed by the kinetics of translation (which we refer to as kinetic effects) or the thermodynamics of the RNC complex (which we refer to as thermodynamic effects). This issue is important because cotranslational folding populations, pathways, structures, and mechanisms can differ significantly depending on whether kinetic or thermodynamic effects predominate. If kinetic effects occur, the cotranslational folding probability will be less than its thermodynamically determined value at one or more nascent chain lengths and the length at which stable domain folding is achieved (defined as $P_F > 0.5$) will be increased (Fig. 1B). Consequently, kinetic effects will tend to delay cotranslational folding as a function of nascent chain length, resulting in an excess population of metastable, ribosome-bound unfolded states.

We address these questions for the cytosolic proteome of *Escherichia coli*. We focus on this organism because it is unicellular, which avoids issues of proteome variation among different cell types. *E. coli* has also been extensively characterized in terms of its genome and proteome, and the concentrations of ribosome and tRNA molecules present at different growth rates have been measured (11), allowing predictive models for $\tau_{A,i}$ to

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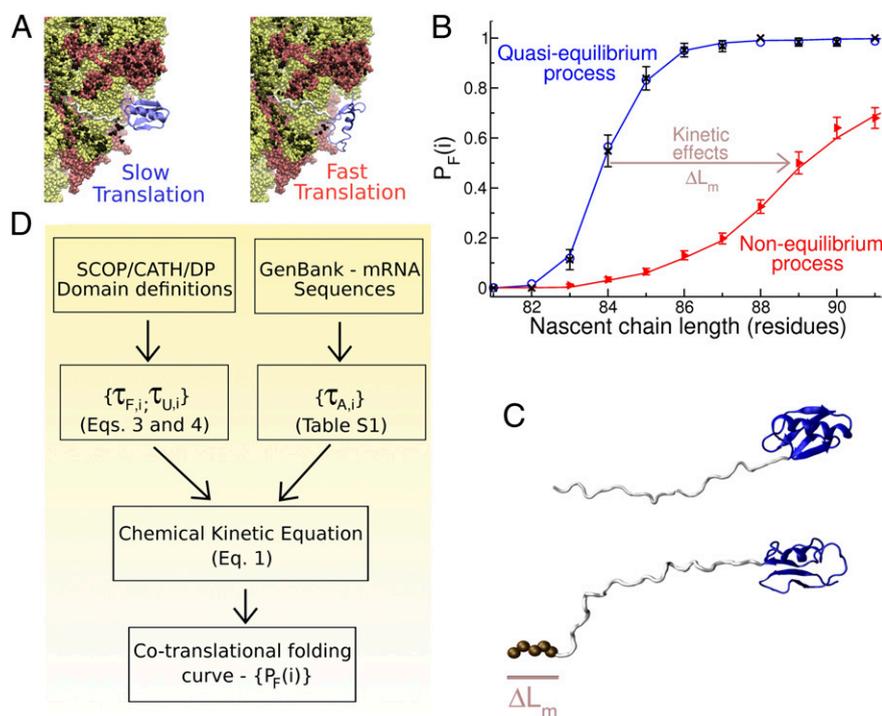


Fig. 1. A systems approach for predicting the cotranslational folding behavior of *E. coli*'s cytosolic proteome. (A) 50S ribosomal subunit with ribosomal protein and RNA molecules shown, respectively, in red and yellow. The protein G domain (blue) is shown emerging from the exit tunnel, and it is tethered to the P-site tRNA by an unstructured poly-glycine linker (white). (B) Cotranslational folding curves ($P_F(i)$) for a protein G domain synthesized at finite translation rates (red triangles, $\tau_A = 1.3$ ms) and at an infinitely slow translation rate (black X symbols). Data were taken from the coarse-grained molecular dynamics simulations reported by O'Brien et al. (7). The deviation between the $P_F(i)$ curves is characterized by ΔL_m , the separation in nascent chain lengths at which the native state becomes stable, that is when $P_F(i) > 0.5$. The simulation results for $\tau_A = 60$ ms are shown as blue circles; at this translation speed, $\Delta L_m = 0$. (C) ΔL_m can be thought of as the additional number of residues (brown spheres) required for a domain to achieve its stable folded structure at finite translation rates (Lower) compared with infinitely slow translation (Upper). Structures were taken from coarse-grained simulations of protein G reported by O'Brien et al. (7). (D) Illustration of the work flow of our systems approach.

be developed (12). We focus on the *E. coli* proteins that are synthesized and remain in the cytosol because this strategy avoids the need to consider additional processes, such as cotranslational protein translocation through translocons (13), that would complicate our approach to modeling and analysis.

Our unique systems approach to these questions combines a kinetic model, which predicts the effect of individual codon translation rates on the cotranslational folding of protein domains (7), with biophysical data on *E. coli* to predict the cotranslational folding curves for protein domains in the *E. coli* cytosol (Fig. 1D). In this way, we have been able to calculate cotranslational folding curves at the rate of translation found in *E. coli* (an out-of-equilibrium process), as well as the curves that would arise from an infinitely slow translation process (a quasiequilibrium process; Fig. 1B). Our results indicate that a number of proteins exhibit cotranslational folding in *E. coli* and that for many of these proteins, translation kinetics, rather than the thermodynamics of the RNC complex, govern their cotranslational folding behavior. This important physical feature of cotranslational folding has a number of significant implications for cellular biology, biotechnology, and synthetic biology, as we discuss below.

Methods

Creation of a Database Characterizing *E. coli*'s Cytosolic Proteome. The wealth of quantitative data characterizing the K12 MG1655 strain of the Gram-negative bacterium *E. coli* enables us to estimate the cotranslational folding properties of its cytosolic proteome. The data we used in this study were drawn from several sources, which we organized into a database (Dataset S1 and see the *SI Appendix* for dataset formatting information). Full details of the construction of this database are provided in *Supplemental Information*. Briefly, for the 4,319 unique coding sequences in the *E. coli* transcriptome,

we identified those that had an X-ray structure in the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (14) with a resolution of less than or equal to 3 Å or an NMR entry, and also had domain definitions in the Structural Classification of Proteins (SCOP) (15) or CATH (16) database. This procedure resulted in 802 proteins. For the 489 additional proteins that had an RCSB structure but no SCOP or CATH entry, we used Domain Parser (DP) (17) to identify their domains; the latter algorithm was able to identify domains in 264 of these proteins, and the remaining proteins were not included in the database. A small percentage of SCOP and CATH domains were reported to be made up of multiple segments. Given that the sequence separation between segments is quite large (Fig. S1), we treated these discontinuous segments as separate domains, provided they were at least 50 residues in length. We then used PSORTb 3.0.2 (18) to classify proteins on the basis of their subcellular localization; PSORTb includes 4,148 entries that correspond to 4,129 Uniprot identification numbers, of which 1,898 are cytoplasmic, 818 are of unknown localization, and 54 fall into a category of unknown/multiple compartments. To maximize the coverage of our database, we assumed that proteins of unknown localization were cytoplasmic. After removing redundant structures that mapped onto the same protein domain, the database consisted of 758 unique proteins containing 1,236 domains, which covers 30% of the proteins that are cytosolic in *E. coli*. For each domain entry in our database, we report its corresponding range of residue numbers in the PDB file, its range of codon numbers along the mRNA molecule, its mRNA sequence, its domain structural classification (mostly α , mostly β , or mixed α/β), and its total number of residues. Furthermore, for each protein, we report its corresponding gene expression level in *E. coli* from several experimental sources. These data were used to inform the kinetic model and the analysis that are detailed below.

Although our database contains only the subset of *E. coli* cytosolic proteins for which structural data are available, we find no systematic difference between the length distribution of proteins in our database and that in the full set of cytosolic proteins (Fig. S2), indicating that our database is representative of the full cytosolic proteome, at least in terms of protein size.

Calculation of the Cotranslational Folding Curve for a Given Protein Domain.

Central to our analysis is the calculation of the cotranslational folding probability of a protein domain as a function of its nascent chain length, referred to as the cotranslational folding curve. We assume the domains in our database are autonomous, cooperative folding units that exist in either a folded or unfolded state with a negligible population of intermediates. For a ribosome that exhibits exponential dwell times at each codon, the ensemble averaged domain folding probability $P_F(i)$ as a function of nascent chain length i is (7)

$$P_F(i) = \sum_{j=1}^i \frac{\tau_{F,j}^{-1}}{\tau_{A,j+1}^{-1}} \prod_{k=j}^i \frac{\tau_{A,k+1}^{-1}}{\tau_{A,k+1}^{-1} + \tau_{F,k}^{-1} + \tau_{U,k}^{-1}}, \quad [1]$$

where $\tau_{F,i}$, $\tau_{U,i}$, and $\tau_{A,i+1}$ are, respectively, the mean times of domain folding, domain unfolding, and amino acid addition at nascent chain length i . In this equation, $P_F(i)$ is the probability the domain is folded immediately before adding the next amino acid (Fig. 1B). The equilibrium cotranslational folding curve $P_F^E(i)$ can be calculated by inserting into Eq. 1 $\tau_{A,i} = \infty$ for all lengths of i , which reduces it to

$$P_F^E(i) = \frac{\tau_{F,i}^{-1}}{\tau_{F,i}^{-1} + \tau_{U,i}^{-1}}. \quad [2]$$

To calculate $\tau_{A,i}$, the mean time of translation of the i th codon in the mRNA sequence, we used the method of Viljoen and colleagues (12), which estimates $\tau_{A,i}$ as a function of the codon identity, and the in vivo concentration of cognate, near-cognate, and noncognate tRNA molecules (SI Methods). This method accounts for competitive binding of cognate and noncognate tRNA molecules for a codon and accurately predicts the average translation rate of various proteins measured in vivo. We used the in vivo concentrations of the ribosome and tRNA molecules measured at five

different *E. coli* exponential growth rates (11) to calculate $\tau_{A,i}$ for each codon at each growth rate (Tables S1 and S2).

To estimate $\tau_{F,i}$ and $\tau_{U,i}$ for a given domain, we fit an empirical scaling relationship to the cotranslational folding and unfolding kinetics of protein G from previously reported coarse-grained simulations (7) of this RNC complex (Fig. 2A). The relationships have the functional forms

$$\tau_{F,i} = \tau_{F,bulk} + a\tau_{F,bulk}e^{-i+l+25} + b\tau_{F,bulk}/i^c, \quad [3]$$

$$\tau_{U,i} = d\tau_{U,bulk} / \left(1 + e^{-i+l+30}\right), \quad [4]$$

where the fitting parameters a , b , c , and d have values of 404, 3205.5, 1.72, and 0.953, respectively. In Eqs. 3 and 4, l is the codon number corresponding to the C-terminal residue of the domain, i is the nascent chain length in number of residues, and c is an exponent whose value is equal to 1.72. The simulations on which the fit parameters were determined used an unstructured poly-glycine linker to attach the protein G domain to the peptidyl transferase center (PTC) (7). We do not expect there to be significant changes in the behavior of these equations when different sequence compositions for the linkers are used.

To estimate $\tau_{F,bulk}$ and $\tau_{U,bulk}$, the folding and unfolding times of the domain when it is free in solution, in Eqs. 3 and 4, we used the De Sancho-Muñoz (DM) model (19), which predicts these two time scales based on the number of residues in the domain, its structural class, as well as the solution temperature (SI Methods). The DM model was chosen over others (20) because it provides an estimate of $\tau_{U,bulk}$, which many other models do not provide, and it also accounts for the effect of temperature on these time scales. The latter is important because we will analyze *E. coli*'s proteome behavior near its optimal cellular growth temperature of 37 °C (21) and most quantitative experimental measurements of bulk folding behavior have been carried out near 25 °C (22). The l , $\tau_{F,bulk}$, and $\tau_{U,bulk}$ values for each domain are reported in Dataset S1.

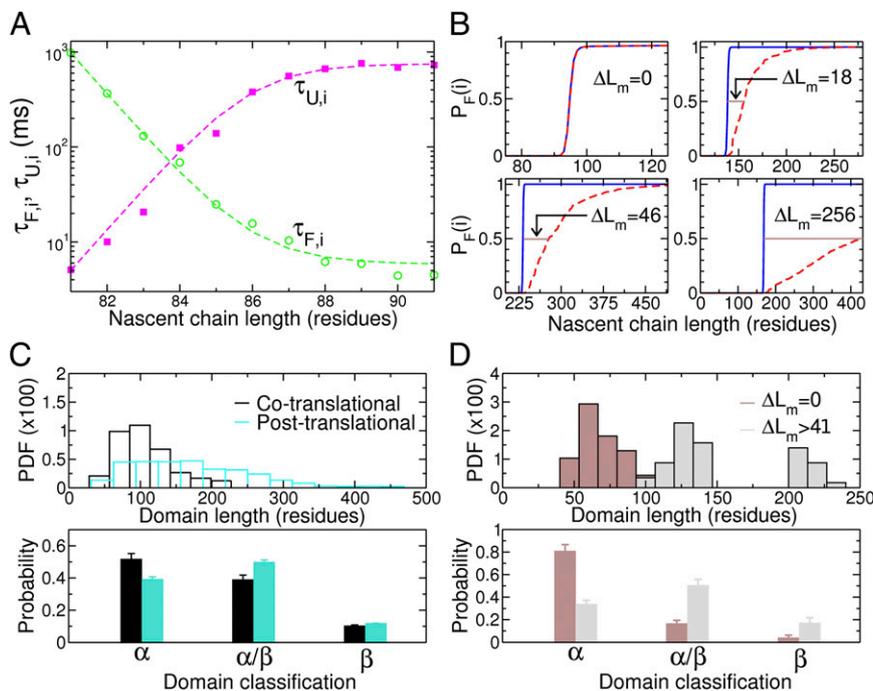


Fig. 2. Cotranslational folding of cytosolic protein domains in *E. coli*. (A) Determination of a scaling relationship to model cotranslational domain folding/unfolding kinetics. The mean folding (green) and unfolding (magenta) times of protein G as a function of nascent chain length calculated from coarse-grained simulations (7) were fitted by Eqs. 3 and 4 (dashed lines). (B) Examples of cotranslational folding curves calculated for four different protein domains in *E. coli* at in vivo (red, Eq. 1) and infinitely slow (blue, Eq. 2) translation rates. The domains correspond, respectively, to (ASNC_ECOLI, domain 1; Upper Left), (3MG2_ECOLI, domain 1; Upper Right), (ILVC_ECOLI, domain 1; Lower Left), and (ENO, domain 1; Lower Right) in Dataset S1. (Upper Left) Note that the red and blue lines are superimposed. (C) Structural characterization of domains that fold cotranslationally and posttranslationally in *E. coli* cells that are dividing every 150 min at 37 °C. (Upper) Probability density function (PDF) vs. domain length. (Lower) Probability of different domain classifications in terms of mostly α (α), mostly β (β), or mixed α/β secondary structure. (D) As in C, except the data are from protein domains that fold cotranslationally with $\Delta L_m = 0$ and those that fold with ΔL_m values greater than 41 residues. The noncontiguous distribution for the $\Delta L_m > 41$ distribution arises from the small number of domains used in its construction ($n = 41$ data points).

$\tau_{F,bulk}$ and $\tau_{U,bulk}$ are functions of a domain's size and structural class in the DM model (19). Therefore, in Eqs. 3 and 4, each domain will exhibit unique $\tau_{F,i}$ and $\tau_{U,i}$ values. The qualitative behavior of these equations is such that as the nascent chain increases in length, the folding time becomes smaller, approaching its bulk value at sufficiently long lengths. Specifically, when more than 28 residues connect the C-terminal residue of a domain to the P-site tRNA on an arrested ribosome, $\tau_{F,i} < \tau_{U,i}$ and the folded state of the domain is thermodynamically more stable than the unfolded state; around 28 residues in linker length, $\tau_{F,i} \approx \tau_{U,i}$ and the domain is at or near its midpoint of stability; below 28 residues, $\tau_{F,i} \gg \tau_{U,i}$ and, consequently, the folded state of the domain is highly unstable, with shorter nascent lengths leading to an exponential increase in the stability of the unfolded state relative to that of the folded state (Fig. 2A).

This behavior is consistent with a wide range of experimental results. A study of GFP found that it is capable of forming a stable native fold on the ribosome when between 22 and 31 residues connect it to the peptidyl-tRNA (23). A cross-linking study found that the midpoint of stability of an α -hairpin tertiary structure was between 24 and 30 residues in linker length (24). A single molecule pulling experiment found that the T4 lysozyme protein was folded at a linker length of 41 residues (25); shorter linker lengths, however, were not probed in that study. A similar upper bound was found in NMR (26) and proteolysis experiments (27), as well as by simulation studies (28). Thus, Eqs. 3 and 4 account for structural differences between domains that result in varied kinetics and exhibit realistic ribosome behavior.

Results

About One-Third of the *E. coli* Cytosolic Proteome Exhibits Cotranslational Folding That Primarily Involves Multidomain Proteins. We treat each SCOP-, CATH-, and DP-defined domain in our database as an autonomous cooperative folding unit that folds in a two-state manner with a negligible intermediate state population. For each cytosolic domain in Dataset S1, we calculated its cotranslational folding curve as a function of nascent chain length at the translation rates found in *E. coli* at 37 °C with a doubling time of 150 min (Fig. 2B). A protein exhibits cotranslational folding if at least one of its domains folds into its native structure with a probability of greater than 0.5 before synthesis of the full-length protein is complete. Under these conditions, we find that about 37% of *E. coli*'s cytosolic proteome (283 unique proteins in our database out of 758) exhibits cotranslational folding, whereas the remaining proteins fold posttranslationally, with none of the domains in these proteins folding during their synthesis.

Substantial differences are found by comparing the properties of proteins that fold cotranslationally and those that fold posttranslationally. The overwhelming majority (91%) of cotranslationally folding proteins are composed of multiple domains, whereas among posttranslational folders, single and multidomain proteins are almost equally represented (51% vs. 49%). In addition, the structural characteristics of those domains that cotranslationally fold are different from those of posttranslational folding domains. Cotranslationally folding domains are smaller in size on average (106 vs. 177 residues; Fig. 2C) and are more likely to be structurally classified as predominantly α -helical ($51 \pm 4\%$ vs. $39 \pm 2\%$ for posttranslationally folding domains; Fig. 2C). On the other hand, posttranslationally folding domains tend to consist of a mixed α/β structure ($50 \pm 2\%$ vs. $39 \pm 3\%$ for cotranslational folders; Fig. 2C). Thus, the structural characteristics of a domain are a factor in determining whether cotranslational folding occurs.

When the growth rate of *E. coli* increases, the rate of translation also increases for various proteins (29). To probe the effect of growth rate on our results, we used biochemical measurements of tRNA concentrations at 37 °C (11, 30) to calculate $\tau_{A,i}$ at different *E. coli* doubling times (Tables S1 and S2) for use in Eq. 1. We find that the percentage of the proteome exhibiting cotranslational folding is insensitive to the change in growth rate, staying near 37% at all doubling times (Fig. S3).

The Majority of Cotranslational Folding Events in the *E. coli* Cytosol Exhibit Kinetic Effects That Can Substantially Delay Folding. Two cotranslational folding curves of a domain can be calculated from the chemical kinetic model described in *Methods*: the folding curve

at the translation rates found in *E. coli* (Eq. 1), where translation kinetics have the potential to decrease the probability of cotranslational domain folding (Figs. 1B and 2B), and the folding curve generated at an infinitely slow translation rate (Eq. 2), where the thermodynamics of the RNC complex determine the cotranslationally folded population. The difference in the midpoint nascent chain length between these two curves, ΔL_m , provides a measure of the deviation from quasiequilibrium behavior exhibited by cotranslational folding in *E. coli* and an estimate of the number of nascent chain lengths at which kinetic effects are exhibited. Another way to interpret ΔL_m is that it represents the additional number of residues necessary to allow a domain to reach its native state due to the finite translation rates in *E. coli* (Fig. 1C). When $\Delta L_m = 0$, the folding curves superimpose (or are at least highly similar) and thermodynamic properties govern the extent of cotranslational folding at each nascent chain length. When $\Delta L_m > 0$, the curves deviate from one another and kinetic

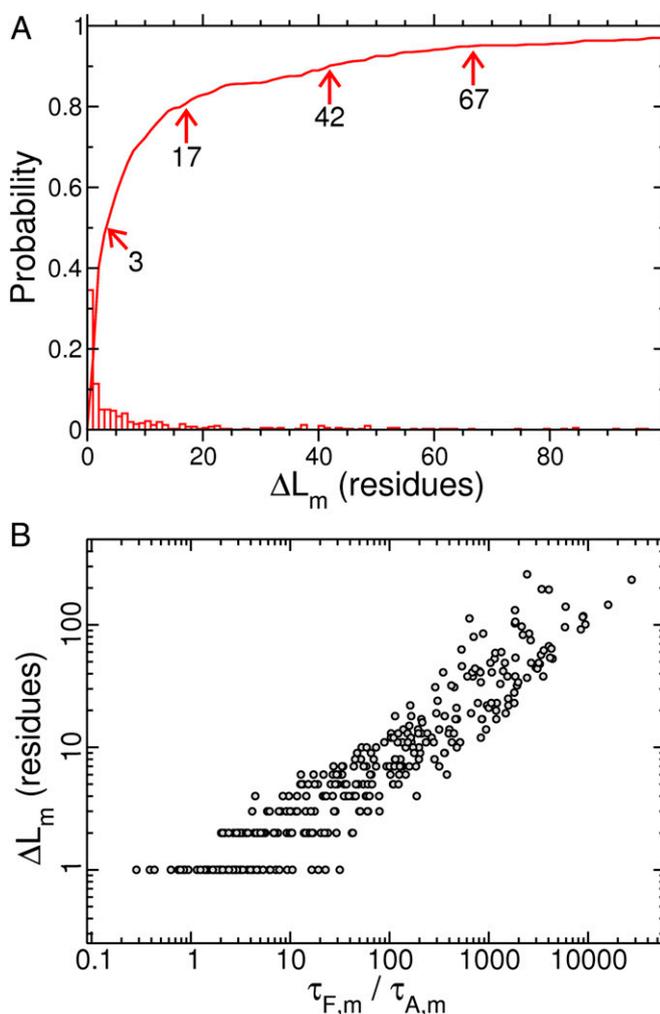


Fig. 3. Extent to which kinetic effects are exhibited during in vivo cotranslational folding and its correlation with the separation in time scales. (A) Probability distribution of ΔL_m values for domains that exhibit cotranslational folding in *E. coli* doubling every 150 min at 37 °C ($n = 422$). The cumulative distribution function (CDF) is shown as a solid red line. The arrows and numbers indicate (from left to right) the ΔL_m values at which the CDF equals 0.5, 0.8, 0.9, and 0.95, respectively. (B) ΔL_m for cotranslationally folding proteins as a function of the ratio of $\tau_{F,m}$ to $\tau_{A,m}$ at nascent chain length m at which the midpoint of domain folding stability occurs at an infinitely slow translation rate.

explored was that ΔL_m correlates with the expression level, but we observe no such correlation in our data (Fig. S5). These results therefore demonstrate that the extent of kinetic and thermodynamic effects is uncorrelated with gene expression levels.

Kinetic Effects Are Exhibited Even When Noncooperative Subdomain Folding Occurs. In the analysis above, we assumed the SCOP, CATH, and DP domain definitions represent autonomous, cooperative folding units. Under this assumption, these domains are only capable of forming a stable, folded tertiary structure once all the residues comprising the domain have emerged from the exit tunnel and are sterically permitted to come into contact, which can include tertiary structure formation in the last 2 nm of the exit tunnel (24, 32). Recent experimental results, however, call this assumption into question because the CATH-defined NBD1 domain of human CFTR protein was found to exhibit stable subdomain folding during synthesis on the ribosome (33). Thus, noncooperative folding can occur on the ribosome, with a stable native tertiary structure forming before synthesis of the entire domain is complete. This phenomenon has the potential to alter the conclusions of this study because subdomains, being smaller, will, on average, fold faster than full-size domains (34). This will change the interplay of the time scales as expressed in Eqs. 1 and 2.

To estimate the robustness of our conclusion of widespread kinetic effects against the potential for subdomain folding, we divided each domain in Dataset S1 that was greater than 150 residues in length into two halves and defined the resulting subdomains as autonomous folding units. This procedure represents an extreme limit in which subdomain folding is widespread among proteins in the cytosolic proteome. As a consequence, the estimated folding and unfolding times on the ribosome are altered (Eqs. 3 and 4). With this new set of domain definitions, we find that the distribution of ΔL_m is shifted slightly toward smaller values (Fig. 4C). The overall trend, however, is highly similar to that in Fig. 3A, and our conclusion that the majority of proteins in the cytosolic proteome exhibit kinetic effects is unaltered by the occurrence of subdomain folding during translation.

Separation of Folding and Amino Acid Addition Time Scales Determines the Extent to Which Kinetic Effects Are Exhibited. A key thermodynamic concept is that the ratio of time scales during an irreversible process determines whether it is a quasiequilibrium process (in which thermodynamics dominate) or a nonequilibrium process (in which kinetics dominate; Fig. 1B). Therefore, we hypothesized that the deviation of a given cotranslational folding curve in *E. coli* from quasiequilibrium (the arrested ribosome case) should be a function of the ratio of the mean folding time to the time of amino acid addition at its midpoint nascent chain length on an arrested ribosome. We plotted ΔL_m as a function of this ratio and observe a trend in which ΔL_m increases with increasing $\frac{\tau_{F,m}}{\tau_{A,m}}$ (Fig. 3B). When $\frac{\tau_{F,m}}{\tau_{A,m}} \approx 1$, we observe ΔL_m is close to 0, and at $\frac{\tau_{F,m}}{\tau_{A,m}} > 1$, we observe $\Delta L_m \gg 0$, with increased $\frac{\tau_{F,m}}{\tau_{A,m}}$ values correlating with increased ΔL_m values. Thus, the ratio of these two time scales is a primary determinant of the extent to which kinetic effects are exhibited during cotranslational folding.

Synonymous Codon Mutations Can Significantly Decrease Kinetic Effects for a Small Percentage of Cotranslational Folding Domains. Synonymous codon mutations alter the mRNA sequence but leave the translated protein sequence unaltered. Synonymous codons can also change the translation rate, which can itself directly affect the extent of cotranslational folding (7, 8, 12). Thus, altering the mRNA sequence such that the slowest translating synonymous mutation is present at each codon has the potential to minimize kinetic effects by decreasing the $\frac{\tau_{F,i}}{\tau_{A,i+1}}$ ratio.

To examine the extent to which kinetic effects on cotranslational folding can be reduced by synonymous codons, we replaced

each codon in each mRNA sequence *in silico* with its slowest translating synonymous codon. We then recalculated the cotranslational folding curves for each domain and calculated the change in ΔL_m (denoted $\Delta\Delta L_m$) between the WT and mutant mRNA transcripts. The majority of domains exhibit a decrease in ΔL_m of no more than 2 residues due to the synonymous codon-induced slowdown in translation (Fig. 5), but larger decreases are observed for a minority of proteins, with 5% of the cytosolic domains exhibiting a decrease in $\Delta\Delta L_m$ of greater than 43 residues. Thus, for the vast majority of cytosolic proteins, synonymous mutations have little effect on the separation of folding curves between quasiequilibrium and finite translation rates; for a minority, however, synonymous mutations can significantly reduce the influence of translation rate on cotranslational folding, and thereby increase the folded population at shorter nascent chain lengths.

Robustness of the Results Against Changes in Cotranslational Folding Time Scale Estimates. The cotranslational folding time of T4-lysozyme on an arrested ribosome has recently been measured at two different nascent chain lengths using laser optical tweezers (LOT) (25). It was found that at a linker length of 41 residues, the value of $\tau_{F,i}$ under a constant tension of 4 pN, is approximately two orders of magnitude larger than in bulk solution, whereas at a linker length of 60 residues, $\tau_{F,i}$ is within one order of magnitude of its bulk value. This trend toward the bulk folding time at longer nascent chain lengths is consistent with our model (Fig. 2A). However, at these experimental linker lengths, $\tau_{F,i}$ is close to its bulk value in our model. We therefore tested whether or not our conclusions are altered by introducing the longer range effect observed in the LOT experiments by reparameterizing the scaling relationship in Eq. 3 to reproduce the trend seen in the experiments. In this model, $\tau_{F,i}$ is two orders and one order of magnitude larger than its bulk folding time at linker lengths of 41 and 61 residues, respectively (Fig. S6A). Using this alternative scaling relationship, we recalculated the cotranslational folding properties of the cytosolic proteins in Dataset S1.

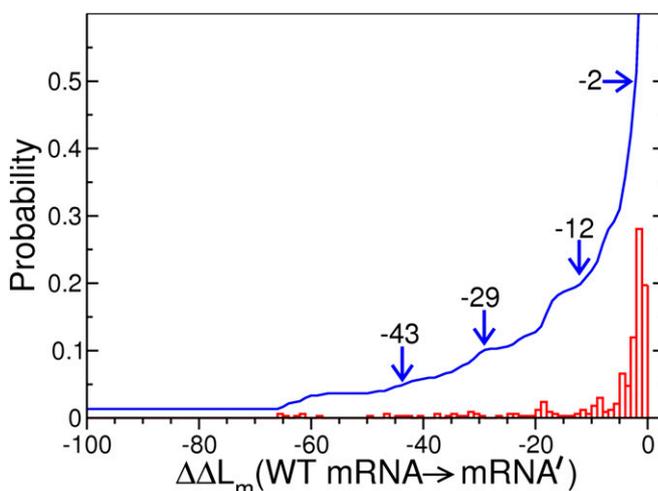


Fig. 5. Slow translating synonymous codon mutations and their affect on the deviation of cotranslational folding from quasiequilibrium. The probability distribution of the change in ΔL_m values for cotranslational folding domains on converting each codon in the WT mRNA transcripts to its corresponding slowest translating synonymous codon is shown. Cotranslational folders that have $\Delta L_m = 0$ for the WT mRNA are not included in this analysis because their $\Delta\Delta L_m$ could never be anything other than 0. The CDF is shown in blue. The arrows and numbers indicate (from left to right) the $\Delta\Delta L_m$ values at which the CDF equals 0.05, 0.10, 0.20, and 0.5, respectively.

We find our results are largely unaltered by this change in $\tau_{F,i}$ behavior. In this model, 37% of the cytosolic proteome is predicted to fold cotranslationally at in vivo translation rates; in addition, 22% of the cytosolic proteins contain at least one domain that switches from co- to posttranslational folding due to in vivo translation rates. Kinetic effects are still observed in a majority of the proteome (Fig. S6B), and the cotranslational folding of α -helical domains remains much more likely to be governed by RNC thermodynamics (Fig. S6C). Thus, our conclusions are robust to changes and uncertainties in the ribosome folding and unfolding time scales.

Discussion

The systems approach presented in this paper (Fig. 1D) offers several important insights into proteome-wide protein folding in the cellular context. With it, we estimate that about one-third of the cytosolic *E. coli* proteome exhibits cotranslational folding (Fig. S3), and we observe that these domains tend to be small and α -helical, whereas posttranslational folding domains tend to be larger and contain a higher β -strand content (Fig. 2C). Further, we find that in *E. coli*, a majority of cotranslationally folding domains exhibit a deviation from their folding curve on an arrested ribosome at one or more nascent chain lengths (Figs. 2B and 3A). This deviation is a manifestation of the nonequilibrium cotranslational folding process that these domains experience during continuous translation. It demonstrates that in vivo translation kinetics, rather than RNC thermodynamics, can determine the folded population of protein domains at one or more nascent chain lengths. As a consequence, the nascent chain length at which these domains reach their stable folded state in *E. coli* can be much greater than that expected under quasiequilibrium (i.e., arrested ribosome) conditions. At the nascent chain lengths that intervene between the midpoints of the quasiequilibrium and nonequilibrium folding curves (Fig. 2B), the folded state is thermodynamically stable but is not populated to the extent that would be expected based on native state thermodynamic stability alone, leading to an excess population of the unfolded state. Thus, the finite rates of translation in *E. coli* reduce cotranslational folding, delay it as a function of nascent chain length, and enrich the population of unfolded nascent chains for a considerable fraction of the cytosolic proteome.

For example, for the domain analyzed in Fig. 2B (Lower Right), the midpoint of stability is shifted by 256 residues as a result of the finite translation rate in *E. coli*. At a nascent chain length of 250 residues, the folding probability of this domain is reduced from ≈ 1.0 on an arrested ribosome to 0.18 on a ribosome translating at in vivo rates, and, conversely, its unfolded state probability is increased from 0 to 0.82. Thus, the predominantly populated unfolded state at this nascent chain length is, in fact, metastable. Remarkably, we estimate that 22% of cytosolic proteins contain domains that would cotranslationally fold if translation were slow enough but do not do so in *E. coli* under exponential growth conditions in rich medium (Fig. S4 and Dataset S2). The reason is that the rate of translation in *E. coli* is fast enough to produce a ΔL_m value for these domains that is greater than that of the full-length protein. Consequently, these domains switch from co- to posttranslational folding, demonstrating that kinetic effects can substantially delay cotranslational folding in vivo.

There are few in vivo experimental data to compare with our predictions of the proteins that fold cotranslationally (Dataset S2). One *E. coli* protein for which convincing evidence exists is the five-domain β -galactosidase protein (3), where the concomitant appearance of the full-length protein and enzymatic activity suggests cotranslational folding is occurring. Our model predicts that this protein exhibits cotranslational folding at in vivo translation rates, with domains 2 and 4 folding before the full-length protein is released from the ribosome and domains 1, 3, and 5 folding posttranslationally. This prediction is consistent with the

experimental conclusion that β -galactosidase folds cotranslationally because the current experimental data do not eliminate the possibility that some β -galactosidase domains may not fold cotranslationally.

The separation in time scales of domain folding relative to amino acid addition determines whether cotranslational folding is a quasiequilibrium or a nonequilibrium process and, consequently, whether thermodynamics or kinetics govern the cotranslationally folded populations. This provides an explanation for the correlation between ΔL_m (a measure of the deviation from quasiequilibrium) and the ratio of the τ_F -to- τ_A time scales (Fig. 3B). At the molecular level, when $\frac{\tau_{F,m}}{\tau_{A,m}} \leq 1$, the domain has sufficient time to equilibrate and reach its global free energy minimum at this nascent chain length. Thus, thermodynamic stability governs the cotranslationally folded population of this domain, and the process is quasiequilibrium in nature. When $\frac{\tau_{F,m}}{\tau_{A,m}} \gg 1$, a domain does not have sufficient time to equilibrate at each nascent chain length because there is a smaller time window for the domain to relax to its global free energy minimum. Under these conditions, kinetic effects suppress the cotranslationally folded population below its thermodynamic value, leading to an enrichment of metastable, ribosome-bound unfolded states. Thus, it is the separation of these time scales that determines the extent to which thermodynamics or kinetics govern cotranslational domain folding.

We have also found that large β -strand-rich domains are more likely to exhibit kinetic effects than small α -helical domains (Fig. 2D). It has been established previously that domain size is an important factor determining the magnitude of $\tau_{F,bulk}$ (34); that is, larger proteins tend to take longer to fold because they have a larger number of residues to incorporate into an ordered state. An additional factor that influences $\tau_{F,bulk}$ is the topological complexity of the native structure, because more complex structures with larger sequence separation between native contacts tend to fold more slowly than domains enriched in local contacts (20). Thus, small α -helical domains will typically have smaller $\frac{\tau_{F,m}}{\tau_{A,m}}$ ratios than large β -strand-rich domains, which means the latter are more likely to exhibit kinetic effects during cotranslational folding than the former. We note that we do not see any codon bias for the different classes of domains, as indicated by a similar average translation rate for the codons following the different domain classes (Fig. S7). This suggests domain topology and domain size, rather than codon bias, is the predominant factor influencing the ratio $\frac{\tau_{F,m}}{\tau_{A,m}}$.

These findings also suggest one reason why it is often difficult to express eukaryotic proteins in *E. coli* (35). Eukaryotic proteins tend to be larger on average than prokaryotic proteins (36); thus, they will normally have larger $\tau_{F,bulk}$ values (19, 34). Furthermore, the translation rate in *E. coli* is up to sevenfold faster than in eukaryotes [10–22 amino acids per second vs. 3–6 amino acids per second (29)], indicating that $\tau_{A,m}$ is smaller in *E. coli*. Thus, for a given eukaryotic protein, its $\frac{\tau_{F,m}}{\tau_{A,m}}$ ratio will be larger when it is expressed in *E. coli* than in a eukaryotic cell. This larger separation of time scales suggests that eukaryotic proteins are more likely to exhibit suppressed cotranslational folding in *E. coli*, which can lead to an increase in the probability that they misfold, aggregate, or are degraded, all of which will decrease their soluble folding yield. These findings also suggest that slowing down the translation rate in *E. coli* will increase successful heterologous expression, a prediction that is consistent with results from experiments in which τ_A was modulated using streptomycin-sensitive ribosomes (37).

Trigger factor (TF) and DnaK are two cotranslationally acting *E. coli* chaperones that preferentially bind to the nascent chains of larger proteins compared with smaller ones (38, 39). In *E. coli* cells in which DnaK and TF were deleted, it was found that larger proteins were much more likely to aggregate than smaller proteins

(38–40). Pulse-chase radio labeling of nascent chains in *E. coli* cells containing nonfunctional chaperone GroEL has demonstrated that such aggregation involves only newly synthesized proteins and not preexisting, folded proteins (41). Together, these data indicate that the nascent chains of large proteins are more likely to interact with DnaK and TF, and to be aggregation-prone. Our results suggest this bias for larger proteins may arise from the increased likelihood that larger domains will remain unfolded on the ribosome and fold posttranslationally. Such posttranslational folding increases the affinity of these chaperones for the nascent chain (42) and affords them more time to interact with the unfolded nascent chain.

A key challenge in systems biology studies is accurately estimating the values for a large number of kinetic parameters. Here, we estimated the various codon translation times using experimental information on in vivo tRNA concentrations (*Methods*). An alternative approach could be to use ribosome profiling data (43), whose measured distribution of ribosome positions on a transcript is a function of $\tau_{A,i}$.

A common experimental procedure to obtain high-resolution cotranslational folding data is to use arrested ribosomes (25, 44). Our results suggest that the folding mechanism in this situation can sometimes differ significantly from that during continuous translation. Transition state ensemble (TSE) properties of the folding domain are influenced by the relative thermodynamic stability of the folded and unfolded states, with the TSE resembling more closely whichever of these two states is less stable [i.e., the Hammond effect (45)]. Because kinetic effects delay folding, and hence cause the unfolded state to be less stable than the folded state once folding does occur (Fig. 2B), it follows that the TSE will shift toward the unfolded state in nascent chains undergoing continuous translation compared with the TSE on an arrested ribosome. Thus, care must be taken when extrapolating protein folding results on arrested ribosomes to the situation on actively translating ribosomes.

The widespread presence of kinetic effects in the cotranslational folding of the proteome suggests that translation-related time scales can be manipulated for synthetic biology, biotechnology,

and cellular engineering purposes to boost (or suppress) cotranslational folding of individual proteins. This possibility has been demonstrated in experiments in vitro and in molecular simulations in which insertion of slow-translating codons at particular points along an mRNA molecule altered the folded population (7, 8). Although synonymous mutations alone may have a significant impact on the cotranslational folding curves of some proteins, we have found that they do not significantly reduce kinetic effects for the majority of the proteome (Fig. 5). Therefore, to alter these individual $\tau_{A,i}$ values to an extent that they affect more of the proteome, higher order processes will need to be considered. These manipulations could include the alteration of mRNA secondary structure (46), the variation in the concentration of charged tRNA molecules in vivo (47), or the introduction of anti-Shine–Dalgarno sequences (48, 49).

In addition to informing strategies for synthetic manipulation of the protein-coding sequences, our results may have implications for understanding sequence changes during evolution. Translational fidelity and its importance to protein structural stability has been proposed as an explanation for the strong relationship between mRNA expression levels and evolutionary rates (50, 51). Mistranslation events, in which an incorrect amino acid is incorporated into the nascent chain, could affect the rates of protein folding and unfolding, and thereby alter the likelihood of co- and posttranslational folding events. The fitness consequences of this could therefore link the cotranslational folding process to the evolution of protein and mRNA sequences.

In conclusion, our approach offers a theoretical framework that can be applied to analyze other cells and tissues in both prokaryotes and eukaryotes, and it has the potential to be used to redesign entire transcriptomes rationally.

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