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Effect of nascent peptide steric bulk on elongation kinetics in the ribosome exit tunnel

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Abstract

All proteins are synthesized by the ribosome, a macromolecular complex that accomplishes the life-sustaining tasks of faithfully decoding mRNA and catalyzing peptide bond formation at the peptidyl transferase center (PTC). The ribosome has evolved an exit tunnel to host the elongating new peptide, protect it from proteolytic digestion, and guide its emergence. It is here that the nascent chain begins to fold. This folding process depends on the rate of translation at the PTC. We report here that, besides PTC events, translation kinetics depend on steric constraints on nascent peptide side chains, and that confined movements of cramped side chains within and through the tunnel fine-tune elongation rates.

Graphical abstract

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Chemical Synthesis of Activated Substrates (see Supplementary Methods).

Keywords

ribosome; translation; nascent peptide elongation; unnatural amino acid incorporation; side-chain dependent elongation rates

INTRODUCTION

The ribosome is endowed with an exit tunnel through which the newborn peptide moves during chain elongation. At one end, the peptidyl transferase center (PTC), translation of mRNA occurs and peptide bonds form. At the other end, the exit port, the elongating nascent chain emerges. In between, the nascent peptide begins to fold^{1–11}. The tunnel, made of rRNA, some protein, water, and ions, plays an active role in early protein folding^{12–14}. The efficiency of this biogenic process depends critically on the rate of translation, which varies as the protein is synthesized^{15–20}, and also depends on the length of a nascent peptide^{8–11, 21}, slow *versus* fast translating codons^{22–26}, mRNA conformations^{27, 28}, and interactions of a nascent peptide with tunnel components and chaperones²⁹. As the peptide wends its way through the ribosome's heterogeneous corridor, ~100Å long and 10–20Å

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wide^{30, 31}, it will encounter distinctive variations and ‘constrictions’. Each amino acid side chain will thus traverse a unique energy landscape along the tunnel walls and will seek a preferred microenvironment, dependent on the physicochemical properties of both the peptide side chain and the tunnel. Any twisting/turning/compaction of the peptide will alter peptide-tunnel interactions, especially in the more confined regions of the tunnel (e.g., the constriction, which is estimated to have a diameter of $\sim 10\text{\AA}$). In the case of so-called arrest peptides, peptide-tunnel interactions relay a signal to the PTC to produce conformational changes that inhibit translation^{32–44}. It is likely that similar sensing and relay mechanisms are used for all peptides, not just for evolutionarily programmed arrest sequences. Besides backbone rearrangements (e.g., kinks and helical turns)^{2–4, 8} of the nascent peptide within the tunnel, we hypothesize that the size¹³, electrostatic properties^{41, 44, 45}, and orientations of side chains, will influence the kinetics of elongation. We predict that these properties of the peptide will contribute to elongation kinetics.

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Clearly the tunnel can accommodate large side chains, introduced either by genetic encoding or by chemical reaction. The former includes unnatural amino acids^{2, 46–50}; the latter includes large cysteine (Cys) reagent molecules, for example, trialkylammonium maleimides that can enter the tunnel and react with nascent peptide cysteines^{13, 51}. Although all of these reagents completely react with cysteines engineered into the nascent peptide, the modification rates of each of these reagents decrease monotonically with their increasing molecular volumes, from 220–440 $\text{\AA}^3$ ¹³. This result supports the idea that covalent modification is kinetically restricted by steric factors within the tight confines of each tunnel location. Moreover, side-chain sterics promote both short-range and long-range rearrangements of the nascent peptide in the tunnel^{13, 14}. While these results suggest a steric component to the modification reactions in the tunnel, they also raise two important questions. First, does the size and nature of a peptide side chain moving within and through the tunnel alter elongation rates? If so, could this be mediated by a feedback mechanism that governs the kinetic and thermodynamic state of the peptide in the P-site, a site at the PTC that binds peptidyl-tRNAs? Second, can the extent of this modulation be accounted for by the physicochemical interactions between peptide side chains and the tunnel walls? To investigate these possibilities, we developed a two-step translation strategy to incorporate natural or unnatural test amino acids into the peptide in the first step, and then further elongate the chain synchronously in a second step. This strategy allowed us to introduce side chains of systematically increasing size and to separate effects on amino acid incorporation from effects on elongation. To determine putative side-chain dependent kinetic effects, we used both natural and unnatural amino acids introduced along the peptide at different tunnel locations. Unnatural amino acids provided the opportunity to systematically alter the size of the side chain with minimum permutation of other properties.

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Our results show that, at sensitive locations within the tunnel, side-chain size inversely correlates with elongation rate, and that the effect can be accounted for quantitatively by van der Waals interactions between the introduced amino acid and the tunnel walls. These studies suggest that peptide-tunnel interactions can modify the energetics and kinetics of physiological processes that underlie conformational rearrangements at the PTC during peptide bond formation.

RESULTS

Arrest and rescue of nascent chains

Native cysteine (Cys) in a nascent peptide can be covalently modified by alkylammonium maleimides (X) while residing in the ribosome exit tunnel¹³. If these alkylammonium moieties were incorporated as unnatural amino acids, X-Cys, and traveled through the tunnel as a peptide is elongated, then we could exploit the systematic increase in size of alkyl groups (methyl, ethyl, butyl) to investigate the effect of nascent peptide side chain sterics on elongation kinetics. Two prerequisites are necessary: i) synthesis of unnatural X-Cys and their corresponding aminoacylated tRNA (X-Cys-tRNA^{Cys}), and ii) a means to selectively incorporate X-Cys and measure the kinetics of subsequent chain elongation. The construct used in all experiments was an mRNA that encodes for a nascent peptide of 173 amino acids⁹ with a single Cys codon at position 123, but lacks a stop codon. Translation was carried out in a cell-free rabbit reticulocyte lysate (RRL) system to generate a nascent peptide that remains attached to the ribosome due to the lack of a stop codon in the mRNA.

To satisfy our first prerequisite, we designed and synthesized a series of trialkylammonium-decorated ethylmaleimides, namely, *trimethylammonium* (TMA), *triethylammonium* (TEA), and *tributylammonium* (TBA), with a range of sizes 220–440 Å³ (Fig. 1). Because maleimides inhibit protein synthesis, these probes could not be used as naked maleimides during translation. Therefore, prior to translation, we covalently linked N-ethylmaleimide (NEM) or NEM decorated with TMA, TEA, and TBA (X) to Cys. These modified Cys could then be attached to tRNA and used in subsequent translation reactions. To synthesize the unnatural amino acid attached to tRNA, we used an *E. coli* tRNA^{Cys} transcript⁵² and a flexible ribozyme for tRNA acylation developed by Suga and coworkers (flexizyme, dFx)⁵³. The flexizyme facilitates aminoacylation of tRNA with amino acid substrates (modified or unmodified cysteine) that have been activated by an attached 3, 5-dinitrobenzyl ester (DBE; Fig. 1). DBE provides a good leaving group, which allows the tRNA aminoacylation reaction to proceed for a wide variety of unnatural amino acids⁵³. The broad substrate range of dFx was optimal for us to use as a catalyst for tRNA aminoacylation. The charging efficiency of each flexizyme reaction ranged from 7–20% for DBE substrates of both modified and unmodified Cys (Fig. S1).

Regarding our second prerequisite to selectively incorporate X-Cys and subsequently determine elongation kinetics, we depleted the RRL of available Cys-tRNA^{Cys} by inhibiting endogenous RRL cysteinyl-tRNA synthetase (CysRS). To inhibit CysRS, we made a nonhydrolyzable aminosulfonamide (Cys-AMS; Fig. 2A), according to the chemical protocols used by Tan and co-workers^{54, 55} (see Materials and Methods). The efficacy and specificity of this Cys-AMS inhibitor were evaluated using purified tRNA synthetases from *E. coli* (*Ec*) or human (*Hs*), specific for cysteine (*EcCysRS*), lysine (*HsLysRS*), glycine (*HsGlyRS*), and alanine (*HsAlaRS*) for the inhibitor concentrations indicated in Figure 2B (*left panel*). The IC₅₀ was below 0.6 μM for CysRS, with little or no inhibition of *HsLysRS*, *HsGlyRS*, *HsAlaRS* up to 40 μM of Cys-AMS, consistent with relatively specific AMS inhibition. During translation of mRNA encoding for a single Cys at residue 123, the presence of Cys-AMS (10 μM), inhibited the endogenous enzyme to give ~ 80% arrest at the

predicted peptide length (Fig. 2B, *top gel*, 'Arr' band, ~ 13 kD for the 122-residue arrested peptide). The mean fraction arrested was 0.82 ± 0.01 (mean $\pm$ SEM, $n = 84$). These conditions provide sufficient signal-to-noise and dynamic range, i.e., separation between Arr and FL bands on the gel, to measure kinetics of elongation from the arrested intermediate to the FL peptide. We conclude that Cys-AMS inhibits the CysRS aminoacylation activity to yield a peptide arrested at the Cys codon. Consistent with this conclusion, a cysteine-free nascent peptide (95 residues⁴) is not arrested in the presence of Cys-AMS nor are any of its residues covalently modified by the cysteine reagent polyethylene glycol maleimide⁵⁶ (PEG-MAL, 5000 Da, SunBio, Korea; Fig. 2B, *bottom gel*).

Rescue of this arrested peptide with exogenously added Cys-tRNA^{Cys} (modified or unmodified Cys) allows a Cys to be incorporated into the nascent peptide at position 123, and chain elongation can proceed to yield the FL peptide (173 residues), with the introduced Cys now 51 residues from the PTC. Rescue of the paused intermediate permits synchronization and measurement of the rate of elongation through the tunnel. Because the tunnel hosts only ~ 33 peptide residues in an extended conformation, the incorporated Cys will eventually arrive outside the tunnel, having traveled the tunnel's entire 100Å-length.

Initially, we added flexizyme-produced Cys-tRNA^{Cys} to rescue translations arrested at the Cys codon (Fig. 3A, lane 2). After 20 min, a full-length peptide (FL, lane 3) was observed (~93% of total protein), with little or no arrested peptide remaining. These data represent almost 10-fold increases in FL peptide compared with FL in the arrested lane ('bleed-through'). To assess whether this rescued FL phenotype had incorporated a Cys, we used a "pegylation" assay⁴ (lanes 4–6). Only a free Cys can be pegylated to give a mass-shifted (~ 10kD) band in the protein gel. Thus, in an uninhibited translation (no Cys-AMS), which synthesizes control FL protein using endogenous native cysteine (Ctl, lane 1), the nascent peptide is pegylated to give a fraction pegylated (Fpeg) of ~ 0.92 (lane 4; uppermost band). Pegylation of the minor FL ('bleed-through') present in the arrested translations gives Fpeg 0.10 (lane 5). In contrast, pegylation of the rescued FL produced by addition of exogenous, flexizyme Cys-tRNA^{Cys} gives an Fpeg of 0.83 (lane 6). These results clearly indicate that a Cys is being incorporated into the arrested peptide, followed by chain elongation to FL. Since the flexizyme reaction does not go to completion but retains some uncharged tRNA^{Cys}, we characterized the effect of the addition of uncharged tRNA^{Cys} alone and a mock flexizyme reaction (containing all reagents except the DBE-substrate) on the stability of the arrested peptide and bleed-through to form FL peptide (Fig. S2B). There is little or no effect of 5 μ M tRNA^{Cys} on either the fraction of arrested peptide (lower band) or on the bleed-through to FL peptide. Thus, in all experiments, we limited levels of exogenously added uncharged tRNA^{Cys} to 5 μ M.

TMA-MAL-Cys-tRNA^{Cys} and NEM-Cys-tRNA^{Cys} also rescue arrested peptide to form FL (Fig. 3B, lanes 3–4). However, in contrast to Cys-tRNA^{Cys} rescue, Fpeg of the rescued FL is 0.03 (lanes 7–8), whereas control FL (Ctl) is maximal (lane 5). Thus, no unmodified (free) Cys is incorporated at the Cys codon when rescue is performed with NEM-Cys-tRNA^{Cys} or TMA-MAL-Cys-tRNA^{Cys}. Similarly, TEA-MAL-Cys-tRNA^{Cys} and TBA-MAL-Cys-tRNA^{Cys} each rescue (Fig. 3C, lanes 3–4), albeit with slower kinetics (i.e., there is still residual arrested species), but again there is little or no pegylation of rescued FL (Fig. 3C,

lanes 7–8). All rescue experiments (Figure 3A–C) were carried out for 20 min in the presence of added exogenous tRNA^{Cys} charged with a natural or modified Cys. Upon longer rescue times for TEA (40 min) and TBA (120 min), rescue is more complete, approaching 80% or 64%, respectively (Fig. 3D; Supplementary Table 1). A summary of maximal incorporation and pegylation of FL protein following addition of exogenous aminoacylated tRNA^{Cys} indicates that modified and unmodified aminoacylated tRNAs both rescue well and similarly, but only peptide rescued with Cys-tRNA^{Cys} is pegylated (Fig. 3D).

Due to the relative increase in size, each synthesized unnatural Cys is expected to exhibit different incorporation and elongation rates. We therefore measured the kinetics of rescue from the arrested pool of peptide (Fig. 3E). To ensure these rates were maximized, we used saturating concentrations of each exogenously added Cys-tRNA^{Cys} and X-Cys-tRNA^{Cys} (see Materials and Methods). In Figure 3E, it is clear that the Cys, NEM, and TMA have similar rescue rates. However, the larger TEA and TBA have, respectively, much slower kinetics, consistent with sluggish incorporation of larger modified cysteines into the arrested peptide.

Intra-tunnel peptide side chains constrain elongation kinetics

These kinetics (Fig. 3E) represent the overall translation rates, which are a consequence of incorporation of each unnatural X-Cys at the PTC as well as each subsequent native amino acid (124–173), and transit of the incorporated X-Cys through the tunnel. The latter includes consequences of peptide-tunnel interactions. To separate these factors and assess how peptide-tunnel interactions affect elongation kinetics, independent of incorporation, we devised the strategy shown in Figure 4A. First, we engineered an amber codon (red box) at residue 118. In the absence of a tRNA with an amber anti-codon, nascent peptide will be prematurely terminated with the amber codon situated at the PTC (Fig. 4A, 1st Step). An amino acid, X, either natural or unnatural, can then be incorporated using X-tRNA^{amber} (suppression, red circle). Incorporation of X amino acid will permit elongation to resume. Second, to segregate incorporation of X from events induced by peptide-tunnel interactions involving X and to synchronize the peptide pool, we engineered a Cys codon (yellow box, middle cartoon) at position 123, five residues downstream from the amber site at 118. Rescue from this arrested site with Cys-tRNA^{Cys} resumes elongation of the suppressed peptide to produce FL peptide (Cys rescue). This strategy permits the previously incorporated X (amber suppression) to traverse most of the exit tunnel. We define the rate of movement from this Cys-arrested site to FL as ρ , and a comparison of this rate for different incorporated X residues highlights the relative effects of different X moieties on the elongation rate. This follows from an experimental design in which the mRNA, tRNAs, and nascent peptide are *identical* except for the introduced residue. In this scenario, unnatural X-Cys is incorporated first at location 118 (red circle) using X-Cys-tRNA^{amber}, after which the peptide elongates and then pauses at the downstream Cys codon (due to the presence of Cys-AMS), thus situating the incorporated X-Cys a few residues from the PTC (Fig. 4A). Further chain elongation of this paused construct with Cys-tRNA^{Cys} allows the previously incorporated X-Cys to traverse most of the exit tunnel. This strategy permits the incorporation of unnatural TMA, TEA, TBA maleimide (X) to finish prior to arrest at the Cys codon and subsequent Cys rescue. Changes in rescue rates (ρ) induced by the upstream

presence of TMA, TEA, TBA (red circle) can then be detected, thus separating size-dependent elongation from incorporation of the unnatural X-Cys at the PTC.

To carry out this strategy, we note that TBA incorporation (suppression) requires a longer time than does TMA incorporation (Fig. 3E) and that all experiments for each unnatural Cys incorporation need to be done under identical conditions (e.g., time of translation, concentration of suppressor tRNA and Cys-tRNA^{Cys}). To accommodate this condition, we chose a 90-min translation/suppression period based on the following results. First, the RRL translation time course, in the presence of Cys-AMS, indicates that peptide synthesis saturates at ~40 min and is relatively stable from 45 to 90 min (Fig. S2A). Second, Cys rescue does not depend on dwell time in the Cys-AMS arrested state because translating for 45, 90, or 120 min prior to rescue with Cys-tRNA^{Cys} resulted in identical rescue kinetics (Fig. 4D; average rescue rate of $0.43 \pm 0.03 \text{ min}^{-1}$ (mean $\pm$ SEM)). Nor does translation, arrest, and rescue over a 4-hour period affect fractional arrest or rescue (Fig. S2C), showing that an AMS-arrested reaction mixture is stable and retains its ability to resume and continue translation to FL. Third, the presence of an amber codon does indeed prematurely terminate translation of the peptide (Fig. 4B, top gel, lane 1, “Amber fragment”) during a translation period of 90 min. Fourth, 90 mins of translation in the presence of *uncharged* suppressor tRNA^{amber} (derived from a “mock” flexizyme reaction without DBE substrate) yields only trace FL (Fig. 4B, top gel, lane 2). In contrast, test suppressors aminoacylated with native Cys (Fig. 4B, top gel, lane 3) efficiently suppress to yield 80–90% FL in 90 min. Fifth, significant incorporation of amino acids occurs through amber codons, e.g., Cys incorporation is highly efficient in a 90-min translation, as determined by >0.82 fraction pegylated (Fig. 4C). Sixth, unnatural X-Cys-tRNA^{amber} produces maximum suppression (~20–30%) by 90 min (Fig. 4B, bottom gels). A saturating level of suppression within 90 min was confirmed for all amino acids and provides reasonable signal-to-noise for suppression to yield FL peptide (Fig. S3A).

To examine the determinants of rescue kinetics without interference from incorporation of X-Cys residues, we carried out a series of two-step translation experiments, according to Figure 4A, using unnatural side chains at positions along the peptide. Initially, we focused on position 118. Incorporation of X-Cys at 118 poised the peptide at the Cys codon at position 123. This was the starting point (time zero) from which to trigger elongation with Cys-tRNA^{Cys} and measure the kinetics of FL appearance (Fig. 5A). Elongation of the peptide from residue 123 to FL causes the unnatural residue at position 118 to traverse ~85 Å of the tunnel, starting in the vicinity above the tunnel constriction and traveling past the exit port to a position outside the tunnel. The elongation kinetics were fit with single-exponential relaxations with rates ρ . There is a rank-order dependence of elongation rate on the size of the side-chain moiety, i.e., Cys>TMA>TEA>TBA. A similar pattern was obtained using a 45 min-translation in the presence of Cys-AMS and suppressor tRNAs (Fig. S3B). Control experiments show that uncharged tRNA^{amber} produces only 2–5% FL under identical conditions of the two-step translation (Fig. S3C, bottom gel). The unnatural side chains of TMA, TEA, and TBA differ from one another conservatively, only by the lengths of their trialkylammonium tails, which allows us to ask whether the effect of side-chain volume V on elongation rate could be due to attractive van der Waals forces between the side chain and the tunnel walls. A simple model supports this possibility (Figs. 5B and S4). Note that the

rate is not linear with side-chain volume, but is exponentially dependent on the effective radius of the side chain. Our results show that the introduced side chains at position 118 interact with the tunnel walls in a size-dependent manner to modulate elongation rate.

While such induction may accompany unnatural side chains, it is intriguing to speculate that analogous size-dependent rearrangements might occur for natural side chains and have consequences for elongation kinetics. To investigate this possibility, we engineered constructs containing a series of consecutive tryptophans and a downstream Cys codon (Fig. 6A). Arrest at this Cys codon was produced by CysAMS. The Trp substitutions were made at residues 120–122, immediately adjacent and upstream to the 123Cys codon, or at 113–115, some distance ($\sim 15\text{\AA}$) from the Cys codon. In both cases, elongation permits the Trps to traverse the length of the tunnel, including the constriction. Rescue with Cys-tRNA^{Cys} exhibits a slowed elongation rate, $0.21 \pm 0.01 \text{ min}^{-1}$ ($n=2$, mean $\pm$ average deviation) and 0.16 min^{-1} ($n=1$), respectively, compared to rescue of a control peptide (no Trps; 120–122 has ProValAsn, 113–115 has SerGlyGly), with a rate of $0.43 \pm 0.02 \text{ min}^{-1}$ ($n=3$, mean $\pm$ SEM; Fig. 6B). In a two-step translation experiment, Trp suppression at 118 was followed by Cys rescue to give an average calculated elongation rate of $0.21 \pm 0.03 \text{ min}^{-1}$ ($n=2$, mean $\pm$ average deviation; Fig. 6C). The control, with Cys-suppression at 118 and subsequent Cys rescue, yields an average rate of $0.35 \pm 0.02 \text{ min}^{-1}$ ($n=3$, mean $\pm$ SEM).

To investigate the elongation rate for a bulky side chain situated initially at different locations along the tunnel, we repositioned the amber codon from 118 to 116, 113, 107, 96, or 72 (7, 10, 16, 27, and 51 residues, respectively, from the Cys codon). This permits the incorporated X-Cys to traverse four different tunnel domains (due to tunnel heterogeneity, dimensions, and water properties) during Cys rescue. Positions 113 and 116 are at the constriction, 107 starts below the constriction, 96 at the vestibule (last $\sim 20\text{\AA}$ of the tunnel), and 72 resides completely outside the tunnel (see Fig. 7). X-Cys, starting at 118 traverses all these domains, albeit possibly at different rates. Both the vestibule and outside compartments are larger and have markedly more bulk-like water than the confined regions deeper in the tunnel^{51, 57}. Peptides with TMA or TBA initially in these compartments (residue 107, 96, and 72) have faster rates of rescue than do TMA- or TBA-peptides rescued from starting positions between the PTC and the constriction (Fig. 7A). This is consistent with movement of the alkylammonium moiety through spatially less confined environments below the constriction. Moreover, the rates for both TMA- and TBA-peptide are similar at these locations. In contrast, rescue rates are markedly slower for TMA- or TBA-peptide incorporated at position 113 and arrested at the constriction before being elongated. Therefore, the results in Figure 7A map out, along the tunnel, both the magnitude of elongation and the relative selectivity of TMA *versus* TBA at the indicated locations.

DISCUSSION

Translation rates are variable and need to be regulated within a prescribed range to optimize accurate and robust folding of the nascent peptide. Although decoding and peptide bond formation at the PTC dictate the kinetics of chain elongation, our results indicate strongly that peptide interactions with the inner walls of the ribosomal tunnel can modulate the rate of elongation. As the nascent peptide moves through the tunnel, it traverses a diverse

landscape of free-energy wells and barriers. Attractive forces between the peptide and tunnel effectively deepen the wells, and steric obstacles to peptide transit erect free energy barriers. In either case, dwell times of peptide side chains in successive binding sites along the tunnel will be enhanced, leading to slowed elongation.

The energetics of side chain-tunnel interactions have been investigated computationally by Pande and co-workers, using molecular dynamics simulations. They showed that disembodied amino acids have different free-energy profiles at different tunnel locations⁵⁸. However, allosteric and electrostatic interactions between connected residues in a nascent chain will modify these energy profiles. Our previous studies have shown that, in response to changes of its primary sequence, the nascent peptide relocates and/or reorients with respect to the tunnel^{13, 14}. It is intriguing to speculate that torsional angles change as a side chain navigates through the confined space of the tunnel. Such gyrations could lead to altered dwell times of peptide side chains at specific tunnel locations, and may contribute to the observed rank-order slowing of rescue detected for TMA, TEA, and TBA. If this is the case, our findings imply that nascent peptides adopt different conformations^{7, 59} or different tunnel pathways during elongation^{2, 40}.

What biophysical factors underlie the rank-order dependence of elongation rate on side-chain size (Fig. 5)? For example, could this phenomenon be explained by electrostatics? A larger-sized alkylammonium has a more delocalized positive charge and therefore decreased electrostatic potential in the vicinity of the side chain. Note that positively-charged side chains cause pausing, i.e., a decreased rate of elongation^{45, 60–62}. Quantitatively, the more cationic side chains in the nascent peptide, the more pausing⁴⁵. If the relative elongation rates manifest in our two-step translation experiments were due to changes in electrostatic potential alone, then one would predict the exact opposite of the results we obtained. Namely, the larger side chains (e.g., TBA) should be associated with faster elongation rates. Our results effectively rule out the possibility that the systematic differences we observe at position 118 are a consequence of the magnitude of a side chain's electrostatic potential. We propose instead that the size and perhaps shape of the side chains underlie the observed quantitative effects on elongation rates. Consistent with this proposal is the excellent fit of our data to a simple model in which an increase in side-chain volume leads to an increased van der Waals attraction between peptide and tunnel, thereby slowing elongation (Figs. 5B and S4).

The influence of altered side chains on elongation rate is most clearly manifest in the tunnel region between the PTC and the constriction, the “upper tunnel” (Fig. 7B). This region is also a critical player in the phenomena of pausing and arrest during elongation^{37, 42, 43}. The sensitivity of this region to steric modulation has two likely explanations. First, the cramped quarters will enhance contact between the peptide and tunnel. Second, its proximity to the PTC will optimize cross-talk between these regions.

Consistent with the above interpretation, when an introduced side chain resides in a wider region of the tunnel below the constriction, there are two consequences: a faster elongation rate ρ , and an impoverished sensitivity to side-chain volume. We show this schematically in Figure 7B. At each of three regions of the tunnel, the elongation rate during rescue is

represented as the sum of two pathways (arrows), a volume-independent (ρ_0) and a volume-dependent (ρ_v) pathway. Thus, the elongation rate may be considered as $\rho = \rho_0 + \rho_v$. Transmission of information from the tunnel to the PTC is likely due to allosteric mechanisms mediated either through the tunnel rRNA, ribosomal protein rearrangements, and/or through the nascent peptide itself (intramolecular contacts within the nascent chain). Regardless of the transmission mechanism, our results suggest that at least one mode of sensing peptide-tunnel interactions involves a steric component in the upper region of the tunnel. Bulkier side chains from either natural (tryptophan) or unnatural amino acids slow elongation rates in the upper tunnel. Moreover, the higher side-chain sensitivity at upper regions of the tunnel implicates a role for Van der Waals interactions and steric modulation in the kinetics, as well as the extent, of genetic incorporation of bulky unnatural amino acids into proteins. This is critically relevant for creating tools for probing structures of proteins and their intra- and intermolecular interactions in biological systems.

Several provocative ideas arise from these studies. First, TMA, TEA, and TBA side chains residing between the PTC and the constriction may induce a conformation of the nascent peptide that slows elongation. Second, prolonged arrest at the PTC may stabilize the peptidyl-tRNA in an inactive conformational state^{63, 64}. Whether resumption of translation from this state is rate-limited by a single event, followed by rapid elongation cycles, is unknown. Finally, dragging a recalcitrant TBA through narrow regions of the tunnel could slow subsequent addition of each amino acid at the PTC. More extensive investigations are required to discriminate among these possibilities.

Movement of the peptide within and through the tunnel is coordinated with decoding and synthetic mechanisms at the PTC, and likely with events at the exit port. These are not separable processes. Our results demonstrate subtle differential modulation of elongation kinetics possibly mediated by van der Waals forces and tunnel location. This is important because translation rates can affect translational fidelity⁶⁵ and the conformational state of a nascent peptide in the ribosome^{6, 17, 18, 24, 66–68}. Altered rates of translation may also switch the biogenic sequence of folding^{69, 70} and alter the final structure and function of a protein^{16, 23, 71–73}. Clearly, each of these sequelae have evolved to optimize biogenesis and to avoid the potentially grievous consequences of misfolding.

MATERIALS and METHODS

Constructs

Standard methods of bacterial transformation, plasmid DNA preparation, and restriction enzyme analysis were used. The nucleotide sequences of all mutants were confirmed by automated cycle sequencing performed by the DNA Sequencing Facility at the School of Medicine on an ABI 377 Sequencer using Big dye terminator chemistry (A0BI). All mutant DNAs were sequenced throughout the entire coding region. Engineered mutations and restriction enzyme sites were introduced into pSP/Kv1.3/cysteine-free⁷⁴ using QuikChange Site-Directed Mutagenesis Kit. This 173-cut construct was derived from the N-terminus of Kv1.3 through to residue 173 (EarI-restriction site)⁹. A single cysteine was engineered at residue 123, 51 amino acids from the PTC in the full-length protein, to give 123C/173. Tryptophan mutations at residues 120–122 and 113–115, replacing native ProValAsn and

SerGlyGly, respectively, were made by standard methods as described above. Amber codons, UAG (5'→3'), were engineered at sites 72, 96, 107, 113, 116, and 118, one at a time, in the 173-residue construct used in all experiments. tRNA was made from *E.coli* cysteinyl-tRNA transcript with a GCA (5'→3') anticodon⁵². Suppressor tRNA ("tRNA^{amber}") was made from *E.coli* tyrosinyl-tRNA transcript containing an amber anticodon, CUA (5'→3')⁷⁵. We prepared synthetic capped cRNA in vitro from EarI-linearized templates using Sp6 RNA polymerase (Promega, Madison, WI). These constructs lacked a stop codon so that the nascent peptide remains attached to tRNA and the ribosome during/after translation of mRNA.

In vitro Translation

Normal translation: Translation reaction mixtures (25µl total) were prepared by combining rabbit reticulocyte lysate (according to the Promega Protocol and Application Guide) and ribonuclease inhibitors (RNasin), in the absence or presence of CysAMS (10 µM final [CysAMS]). This mixture was incubated for 10 minutes at 22°C. The purpose of this pre-incubation was to inhibit any endogenous CysRS in the translation mixture. After preincubation, [³⁵S]Methionine (2µl/25µl translation mixture; ~10 µCi/µl Express Dupont/NEN Research Products, Boston, MA) and 2 µl of a 0.4µg/µl mRNA for the 123Cys/173 construct were added to the reaction mixture and incubated for 45 min (Figs. 3, 6B, S2B), 90 min (Figs. 4B, 4C, 5, 6C), or as otherwise indicated in the Figures. This time was optimal for maximizing the amount of final nascent protein in the translation reaction (signal-to-noise), which saturates and is constant from 45 to 90 minutes of translation. Translation reactions (25 µl) were added to 500 µl phosphate-buffered saline, PBS*, comprised of PBS, Ca-free, containing 4 mM MgCl₂, 2 mM DTT, pH 7.3, and centrifuged at 70,000 rpm/20 min/4°C (TLA 100.3 Beckman rotor) through a sucrose cushion (120 µl, containing 0.5 M sucrose, 100 mM KCl, 50 mM HEPES (NaOH or KOH), 5 mM MgCl₂, 2 mM DTT, pH 7.5). Pellets were resuspended in 30µl of LDS NuPAGE Sample Buffer, RNase/DNase-free (Sigma-Aldrich, cat.# 11119915001, final concentration of 20 ng/µl), and incubated at room temperature for 30 minutes.

Suppression at the amber codon: Constructs containing an amber codon were translated as described above for the time specified in each experiment, but in the presence of Cys-tRNA^{amber}, unnatural X-tRNA^{amber}, or Trp-tRNA^{amber}. RF1 releases a portion of the peptide into the supernatant at the amber codon, consistent with premature termination. To confirm/quantitate premature termination at the amber codon, we assayed both pelleted ribosomal-nascent peptide complexes and their supernatants. Supernatants were precipitated with cold acid-acetone, stored at -80 °C for a minimum of 2 hours, centrifuged to separate pellets from supernatants, then resuspended in loading buffer and fractionated on NuPAGE gels. Prematurely terminated peptide fragments in the supernatants ranged from 50–70% of total amber peptide fragments in the translation reaction. Trp-tRNA^{amber} was made by a flexizyme reaction using activated Trp-cyanomethyl ester (CME; see Supplementary Methods. All other activated substrates (Cys, TMA, TEA, TBA) were made as DBE derivatives (see Supplementary Methods).

Rescue of arrest at the cysteine codon: Translation, as described above, followed by addition of aminoacylated tRNA^{Cys} (Fig. 3E): 0.4–2.2 (Cys), 0.25–0.5 (TMA), 0.2–0.4 (TEA), and 0.34 (TBA) μ M, saturating concentrations for incorporation. In protocols where an amber codon preceded the Cys codon, flexizyme-made Cys-tRNA^{Cys} was added to the reaction after 90 min (or otherwise indicated time) of translation in the presence of suppressor X-tRNA^{amber}. In this case, suppressor X-tRNA^{amber} or Cys-tRNA^{amber} was added 5 min after the translation reaction was started.

Determination of CysAMS inhibition constant

Purified *E. coli* CysRS (10 nM) was tested for aminoacylation of the purified transcript of *E. coli* tRNA^{Cys} (10 μ M) with ³⁵S-Cys (25 μ M) at 37°C. Aminoacylation was corrected for a no-tRNA control and quantified by measuring acid-precipitable counts of ³⁵S-Cys-tRNA^{Cys} on filter pads^{76, 77}. The fraction aminoacylated was measured at steady-state, allowing for multiple turnovers of the enzyme, using a saturating tRNA concentration. Thus, the time course of aminoacylation could be fit to the equation $y = ax + b$, where a is the rate constant, k_{cat} , and b is the intercept with the y axis. The aminoacylation k_{cat} was measured at each concentration of the inhibitor Cys-AMS for *Ec*CysRS (freshly prepared), *Ec*CysRS (freeze-thaw once), *Hs*AlaRS (human alanyl-tRNA synthetase), *Hs*GlyRS (glycyl-tRNA synthetase), and *Hs*LysRS (lysyl-tRNA synthetase), each at 10 nM.

Chemical Synthesis of Charged tRNA

Preparative Flexizyme Protocol: A mixture of equimolar amounts of tRNA and dFx flexizyme in Hepes buffer (pH7.5) was incubated at 90°C for 2 minutes to denature tRNA and flexizyme. After 2 minutes, additional MgCl₂ (10mM) was added and the reaction equilibrated at room temperature for 5 minutes. This solution was placed on ice and supplemented with 3 M MgCl₂ and 25 mM DBE-activated amino acid in DMSO to give a reaction mixture of 333 μ L that contained 38 μ M tRNA, 38 μ M dFx flexizyme, 4 mM aminoacyl DBE, 390 mM MgCl₂, 120 mM Hepes, pH 7.5, and 20% DMSO. Incubations were carried out in an ice bath using optimized times derived from the analytical reaction (below) for each DBE reagent. Reactions were quenched with an equal volume of 0.6 M NaOAc, pH 5, precipitated with 70% ethanol for 1 hour at –80°C, and centrifuged at 14,000 rpm (Eppendorf centrifuge) for 30 minutes at 4°C. Pellets were washed with 70% ethanol and resuspended in 50 μ L of 25mM NaOAc, pH5, and stored at –80°C. A similar protocol was used for flexizyme charging with Trp-CME.

Analytical Flexizyme Protocol: Analytical flexizyme reactions were carried out to determine the optimal reaction time for each DBE activated amino acid. These reactions, which were 10–20 μ L in volume, followed the same protocol as above but with 5 μ M radiolabeled tRNA^{Cys} (or tRNA^{amber}). The 3'-³²P end-label in this tRNA was introduced in an exchange reaction catalyzed by CCA adding enzyme in the presence of α -³²P-ATP (3000 Ci/mmol, PerkinElmer Health Sciences, Inc., CT). Control experiments demonstrated that the kinetics and extent of tRNA charging were unchanged by this modification. After initiation of the flexizyme reaction 1.0 μ L aliquots were transferred to 9 μ L quench solution (50 mM NaCl, 200 mM NaOAc pH 5) at 15–30 min intervals and stored at –20 °C. For analysis of charging efficiency, 4 μ L of each quenched sample was incubated 20 min at

37 °C with S1 nuclease to generate 3'-mononucleotides. Labeled AMP was resolved from labeled aminoacylated-AMP by thin layer chromatography on a 10 cm long PEI-cellulose sheet run in 5% HOAc/0.1M NH₄Cl. Radioactive spots were resolved by phosphorimaging and quantified using ImageQuant.

Pegylation

Translation reactions were centrifuged (70,000 rpm/20 min/4°C; TLA 100.3 Beckman rotor). The pellets were re-dissolved in 25µl of 1% SDS in PBS* containing 1 mM DTT and 20 ng/µl RNase and incubated at room temperature for 30 minutes. An equal volume of PEG-MAL (95% purity, 5000 kD, SunBio, Korea) was added to give a final solution of 2 mM PEG-MAL. This mixture was incubated at 4°C for 2 hours, precipitated with a 95% final volume of cold acid-acetone (900µl; a stock acid-acetone solution was made by adding 10 µl of HCl to 25 ml of acetone), and left at -80°C overnight. The following day, the precipitated protein was centrifuged at 14,000 rpm (Eppendorf centrifuge) at 4°C and the supernatant removed. The final precipitated sample was mixed with NuPAGE sample buffer and fractionated using SDS-NuPAGE electrophoresis (see below). The fraction of protein pegylated (F_{peg}) was calculated as the ratio of counts per minute in the pegylated band to the sum of the counts per minute in the pegylated and unpegylated (FL) bands. The probability of an unmodified cysteine being present, P_{cys} , is calculated as the observed pegylated fraction of protein divided by the probability that a residue is an available cysteine and gets pegylated (i.e., the efficiency of pegylating a cysteine (control cysteine)).

Gel Electrophoresis

All final samples were treated with ~1µl of 500 µg/ml RNase for 30 min at room temperature to digest tRNA and remove contaminating peptidyl-tRNA bands. Samples were then mixed with LDS NuPAGE sample buffer, 53 mM DTT, deionized water, and heated at 70° C for 10 min prior to being loaded onto the gel. Gels were precast Bis-Tris 10% NuPAGE gels and electrophoresis was run with NuPAGE MES SDS Running Buffer for 45 minutes at 180V. Gels were then exposed to gel fixative followed by Amplify (Amersham Corp., Arlington Heights, IL) to enhance ³⁵S fluorography, dried, and exposed overnight to a cassette screen for PhosphorImaging. Quantitation of gels was carried out directly using a Molecular Dynamics (Sunnyvale, CA) Typhoon FLA 9500 PhosphorImager. Because full-length protein contains 4 methionines whereas Cys-arrested proteins contain 3 methionines, radioactive counts in each band were corrected accordingly.

Chemical Synthesis of Cys-AMS—The synthesis of Cys-AMS was conducted as described in a report by Tan and coworkers⁵⁴. Of note, sulfamoyl chloride, a necessary reagent for this synthesis, was also made in-house according to another literature procedure⁵⁵. All characterizations matched those of prior reports^{54, 55}. Cys-AMS inhibitor was stored in an environment that was both reducing and at neutral pH to prevent dimerization, 45 µg of Cys-AMS was dissolved in 100 µl of fresh buffer stock (10 mM HEPES buffer, pH 7.3, 2 mM β-mercaptoethanol). The stock was diluted with buffer stock to reach a final [CysAMS] of 100 µM and stored at -80°C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- 1.** A systematic increase of side-chain size induces rank-order slowing of elongation rate in upper, but not lower, tunnel regions.
- 2.** Increased side-chain volume leads to increased van der Waals interactions between nascent peptide and ribosome tunnel.
- 3.** A two-step translation strategy was developed to evaluate the effects of bulky side chains on elongation kinetics.

Figure 1. Activated unnatural amino acid substrates

Chemical structures of activated substrates for subsequent use in the flexizyme reaction scheme to synthesize decorated-maleimide Cys-tRNA^{Cys}. Dinitrobenzyl esters of Cys (Cys-DBE), N-ethyl maleimide Cys (NEM-Cys-DBE), trimethylammonium maleimide Cys (TMA-MAL-Cys-DBE), triethylammonium maleimide Cys (TEA-MAL-Cys-DBE), and tributylammonium maleimide Cys (TBA-MAL-Cys-DBE) are shown and used subsequently in the flexizyme reaction to produce either Cys-tRNA^{Cys} or X-Cys-tRNA^{Cys}, where X represents substituted maleimide.

Figure 2. Cys-AMS, a synthetically made inhibitor of CysRS

A. Structure of Cys-AMS. **B. Left:** Steady-state aminoacylation rate constants, k_{cat} , for isolated *E. coli* (*Ec*) and human (*Hs*) synthetases (RS): *EcCysRS* (freshly prepared), *EcCysRS* (freeze-thaw once), *HsAlaRS* (alanyl-tRNA synthetase), *HsGlyRS* (glycyl-tRNA synthetase), and *HsLysRS* (lysyl-tRNA synthetase), each at 10 nM. **Right, Top gel:** Inhibition of nascent peptide elongation in a RRL translation system. Translation was carried out in either the absence (lane 1, control) or presence (lane 2, arrested) of Cys-AMS (10 μ M) and fractionated on a NuPAGE gel. The upper band is the FL peptide and the lower band is the peptide arrested at the Cys codon (Arr), each migrating at its predicted mass (13 and 18kD, respectively). **Bottom gel:** A peptide, similar to that shown in the top gel, but containing no Cys, was translated and fractionated on a NuPAGE gel to give the predicted mass (lane 1) but no arrest after treatment with Cys-Ams (10 μ M, lane 2). Treatment of both products with PEG-MAL gave no gel-shift (lanes 3–4), indicating that no modifiable cysteines were present. Additionally, we have carried out arrest on the same 173-residue peptide with a single Cys at residue 149, a C-terminally extended (201 amino acids) and an N-terminally truncated (51 amino acids) version of this peptide with C149, and on a 117-residue peptide with a Cys at residue 74. Arrest occurs in all cases at the Cys, albeit with different efficiencies.

Figure 3. Incorporation of unnatural cysteines

A–C. Rescue of arrested nascent peptide with the indicated flexizyme-made Cys-tRNA^{Cys} or X-Cys-tRNA^{Cys} for 20 min. Subsequent pegylation of SDS-treated samples (blue-bracketed lanes) are shown to the right of rescue lanes. Lane 1 shows translation of peptide in the presence of *endogenous* native Cys-tRNA^{Cys} (Ctl), whereas translations in the presence of Cys-AMS (lane 2, Arr) give rise to ~85% arrest at the Cys codon. Ctl FL protein shown in lane 1 has a free Cys, as verified by its pegylation: F_{peg} ~0.9 (lane 4 in A, lane 5 in B–C). Addition of *exogenously* made Cys-tRNA^{Cys} or X-Cys-tRNA^{Cys} for 20 min rescues arrested peptide to produce FL peptide (lane 3 in A, lanes 3–4 in B–C). Pegylation of each of these species (blue-bracketed lane 6 in A, lanes 7–8 in B–C) reveals which peptides contain modifiable cysteines. Cys-rescued peptide (A., lane 3) is strongly pegylated (A., lane 6). By contrast, an incorporated unnatural X-Cys (NEM, TMA, TEA, TBA; lanes 3–4, B–C) gives little or no pegylation (lanes 7–8, B–C). Arrested protein (lanes 2 in A–C) is not pegylated (lanes 5 in A, 6 in B–C). **D.** Maximal incorporation and pegylation of unnatural modified cysteines. Translations arrested with Cys-AMS were rescued with the indicated aminoacylated tRNA (X-Cys-tRNA^{Cys}) for times that gave maximum FL protein (F_{incorp}, black bars) and subsequently pegylated (F_{peg}, dark blue). The probability of an unmodified cysteine being present, P_{cys}, is shown as light blue bars (see Methods). Data are means ± SEM for triplicate experiments. **E.** Kinetics of formation of FL peptide upon rescue with flexizyme-generated aminoacylated tRNA^{Cys}. Translations were carried out in the presence of Cys-AMS to give arrested populations, followed by addition of exogenous Cys-tRNA^{Cys} (gray), NEM-Cys-tRNA^{Cys} (yellow), TMA-MAL-Cys-tRNA^{Cys} (green), TEA-MAL-Cys-tRNA^{Cys} (orange), and TBA-MAL-Cys-tRNA^{Cys} (red), and sampled at the indicated times. Solid curves are single-exponential fits to the data. Rates are 0.43 ± 0.02

(Cys), 0.52 ± 0.05 (TMA), 0.37 ± 0.03 (NEM), 0.11 ± 0.01 (TEA), and 0.030 ± 0.003 (TBA) min^{-1} , respectively. These experiments are representative of triplicate repeats. Dose-response curves established the concentration range (0.2–2 μM) of unnatural aminoacylated tRNA^{Cys} used in all experiments and for which incorporation was saturating.

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Figure 4. Site-specific pre-incorporation of test amino acids (Suppression)

A. Strategy for two-step translation to measure the rate of Cys rescue, ρ . An mRNA was engineered with a single amber codon (red box) and a single Cys codon (yellow box) at position 123. Translation of this mRNA yields a peptide fragment at the amber codon (left, 1st Step). Addition of X-Cys-tRNA^{amber} leads to incorporation of X-Cys (red circle), i.e., suppression, elongation of the peptide, and arrest at the Cys codon (yellow box, 2nd Step). Subsequent addition of Cys-tRNA^{Cys} produces incorporation of Cys at position 123 (yellow circle) and elongation to FL peptide (Cys rescue). The rate of this Cys rescue is depicted by ρ . **B.** Kinetics of suppression of mRNA (containing a single amber codon at position 118 and a single Cys codon at position 123) by each indicated suppressor tRNA^{amber}. **Top:** Controls: In the absence of Cys-AMS, 90-min translation alone (lane 1) or in the presence of uncharged tRNA^{amber} from a “mock” flexizyme reaction (no DBE substrate, lane 2), or in the presence of Cys-tRNA^{amber} (lane 3). **Bottom:** Translation of mRNA (in the absence of Cys-AMS) in the presence of the indicated amber suppressor (TMA, TEA, TBA) for the indicated times. Cys incorporation was due to endogenous Cys-tRNA^{Cys}. **C.** Cys incorporation determined by pegylation. Translation (90 min) of a construct containing a single amber codon at position 118 and a single Cys codon at position 123, in the absence of Cys-AMS and in the presence of Cys-tRNA^{amber} (lane 1). FL thus contains two cysteines, one at 118 and one at 123. Translation product was then treated with 2mM PEG-MAL to give singly (Peg 1) and doubly (Peg 2) pegylated protein (lane 2). Total fraction of protein

pegylated is 0.82. **D.** Rescue kinetics of Cys-arrested peptide. Translation was carried out in the presence of Cys-AMS and rescued after 45 (black), 90 (blue) or 120 (red) min with flexizyme-made Cys-tRNA^{Cys}. The FL fraction was calculated as the ratio of FL band intensity to the sum of intensities for FL and Arr. Data were fit with a single-exponential function to give 0.41, 0.46, and 0.41 min⁻¹, respectively.

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Figure 5. Elongation kinetics of suppression-modified nascent chains

A. Time course of Cys rescue. The fraction of FL peptide was monitored following addition of Cys-tRNA^{Cys} to the X-Cys (at 118) peptide arrested at codon 123 (Cys-AMS in the translation (90 min) mixture). *Inset:* Gels display both FL peptide and an Arr band for the indicated times after Cys-tRNA^{Cys} addition. The Arr band contains both prematurely terminated peptide at amber and peptide arrested at the Cys codon. Data were fit to a single-exponential decay. These experiments are representative of triplicate repeats. **B.** Rate (ρ) of elongation to FL versus side-chain volume for peptides with tunnel-resident unnatural Cys side chains at position 118. The data clearly indicate that larger side chains reduce elongation rate. The blue theory curve is the fit of a simple model (see Fig. S4) in which the volume-dependent interaction energy between different side chains and the tunnel is accounted for by van der Waals forces. Data are means $\pm$ SEM for triplicate experiments.

Figure 6. Elongation kinetics of tryptophan nascent chains

A. Scheme for suppression and Cys rescue, ρ , of Trp mutated nascent chains. An mRNA was engineered with a Cys codon (yellow box) at position 123 and consecutive Trps (red oval), either at position 120–122 or 113–115 in the nascent peptide. **B.** Rescue kinetics of Cys-arrested peptide. Translation (45 min) was carried out in the presence of Cys-AMS, for nascent peptide containing 3Trps at 120–122 (magenta) or at 113–115 (cyan) and rescued with flexizyme-made Cys-tRNA^{Cys}. Control peptide (black) is WT with native, non-Trp residues in the nascent chain. The normalized FL fraction was calculated as the ratio of FL band intensity to the sum of intensities for FL and Arr from the gels shown in the inset. Data were fit with a single-exponential function to calculate the elongation rates: 0.41, 0.20, and 0.16 min⁻¹ for WT, 3Trps (120–122), and 3Trps (113–115), respectively. **C.** In a two-step translation experiment, Trp suppression at 118 for 90 min was followed by Cys rescue (as in B.) to give an average calculated elongation rate of 0.21 ± 0.03 min⁻¹ ($n=2$, mean $\pm$ average deviation). The control is a Cys-suppression at 118, followed by Cys rescue to give an average rate of 0.35 ± 0.02 min⁻¹ ($n=3$, mean $\pm$ SEM).

Figure 7. Zones for location-dependent modulation of elongation rate ρ

A. Rates of Cys rescue (ρ) for six peptides, each with TMA or TBA incorporated at the indicated residue (See Fig. 7B for approximate location of these residues along the tunnel). Samples treated as in Fig. 5. Data are means $\pm$ average deviation for duplicate 116, 113, 107, 96, or 72 experiments. The rates for 118 X-Cys were calculated from triplicates as in A (means $\pm$ SEM). Average Cys rescue rates for TMA (green squares) at positions 118, 116, 113, 107, 96, and 72 were 0.27 ± 0.03 , 0.15 ± 0.04 , 0.09 ± 0.06 , 0.34 ± 0.01 , 0.60 ± 0.01 , 0.38 ± 0.09 min⁻¹, respectively. Average Cys rescue rates for TBA (red squares) were 0.11 ± 0.02 , 0.10 ± 0.03 , 0.08 ± 0.02 , 0.38 ± 0.02 , 0.58 ± 0.02 , 0.39 ± 0.11 min⁻¹, respectively. The rates were not significantly different for TMA versus TBA at positions 116, 113, 107, 96, and 72 ($P > 0.20$; ANOVA), except for residue 118 ($P < 0.01$). The locations of the amino acid residues shown on the x-axis are not drawn to scale. **B.** Schematic of nascent peptide-tunnel complex (modified after Wilson and Beckmann, Fig. 2¹²) with peptide (black line) arrested at the PTC at Cys codon 123 (large circle, Arr). The large ribosome subunit (gray) is divided into 3 regions (dashed blue lines), in accord with elongation rates shown in Fig. 7A. The overall rate of exit from this arrested state is depicted as the sum of a volume-dependent (ρ_v) and a volume-independent pathway (ρ_0). The relative length of the arrows indicates the relative contribution of the pathway to the elongation rate. In the upper portion of the tunnel (118), steric modulation slows elongation; at the constriction (116, 113), slow elongation is due to the confined space with no steric modulation. In the mid-region of the

tunnel (107), the vestibule (96), or outside the tunnel (72), rates are fast in the absence of steric modulation.

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