Following Translation by Single Ribosomes One Codon at a Time

Supplementary Information

Successive rate determining steps

For two successive rate-determining steps with different rate constants, the probability of observing a dwell time, τ, depends on the difference of the two rate constants.

$$dP(\tau) = (\frac{k1k2}{k2-k1})(e^{-k1\tau} - e^{-k2\tau})d\tau.$$

If the rate constants are equal the equation reduces to a Poisson equation.

Poisson equations

For successive rate-determining steps with equal rate constants, k, the probability of observing a dwell time, τ, is a Poisson equation. A single step gives a single exponential

$$\mathrm{d}P(\tau) = k \exp(-k\tau)\, \mathrm{d}\tau,$$

two steps require

$$dP(\tau) = (k^2\tau)(e^{-k\tau})d\tau,$$

and three steps require

$$dP(\tau) = (\frac{k^3\tau^2}{2})(e^{-k\tau})d\tau.$$

Translation of S3hp

The 60-bp S3hp hairpin (Fig. 1a) was constructed from the mRNA of the ribosomal protein S3. The presence of unique codons in the mRNA made it possible to stall the ribosome at distinct positions by omitting the corresponding aminoacyl-tRNAs. Translation by the ribosome to a stall position results in partially opening of the hairpin; the remaining size of the hairpin depends on where the ribosome is stalled. Thus, we can tell if the ribosome can unwind the unusually long hairpin and stop at expected positions by measuring the hairpin size. This was done by attaching the ends of the stalled complexes to two polystyrene beads on optical tweezers (Fig. 1, left panel). The force between the beads, and thus on the ends of the hairpin, was gradually increased by pulling the pipette bead away from the trap bead (Fig. 1, left panel). When the force is raised to a critical value (20 – 23 pN in this case), the hairpin unfolds completely in one fast step (on a timescale of less than a second), resulting in a sudden increase in the end-to-end distance (extension) of the construct, as shown in Supplementary Fig. S1a. This sudden change in extension is termed a "rip", which is a measure of the hairpin length. Supplementary Fig. S1a shows a series of unfolding transitions for stalled complexes at

different positions on S3hp. The rip decreased from ~ 30 nm when the ribosome was stalled just before the base of the hairpin (codon R11, see Fig. 1a), to essentially zero when the ribosome was stalled close to the end of the hairpin (codon D31). These experiments amount to snapshots of the translation process.

The stalling reactions in the above experiments were done in bulk, and then stalled complexes were analyzed with optical tweezers. To follow translation on a single S3hp hairpin, a complex with the ribosome stalled at codon R11 was assembled on optical tweezers as described above. A translation mixture containing all the required aminoacyl-tRNAs and elongation factors (see Methods) was injected into the chamber to resume elongation. Evidence of translation on the tethered mRNA was indicated by gradual shortening of the hairpin as the ribosome translocated forward. This was determined by quickly pulling the hairpin to measure the rip size, and then lowering the force to let the hairpin refold. The unfolding-refolding cycles were repeated every 0.5 – 2 min to follow translation on the same mRNA. As shown in Supplementary Fig. S1b, the hairpin did decrease in size as translation proceeded. These experiments established that an RNA duplex can be unwound by the translating ribosome at the single-molecule level.

The overall translation rate in Supplementary Fig. S1b is slow, about 1 codon/min. The rip size decreased by ~ 20 nm (corresponding to translation of 7 – 8 codons) in 8 min (not including 2 min for injecting the translation mixture to fill the chamber). In this example, the ribosome did not translocate on the hairpin in a constant rate. For example, there was a long pause between t = 300 and 450 s, but the rip decreased by 6 nm in the last 30 s (Fig. S1b). These occasional long pauses were observed for most reactions with the same mRNA and also for the other two hairpins (VE60hp and VE274hp), as shown in Fig. 3a (for VE274hp). The causes for long pauses are not clear, but the force applied to the ends of hairpins plays a role, as shown in Fig. 4b. In addition, the reaction mixture used for S3hp contained a mixture of total aminoacyl-tRNAs (see Methods). A longer pause may occur at a codon with a lower concentration of the corresponding aminoacyl-tRNA, due to low-efficiency of aminoacylation or faster hydrolysis. These effects are difficult to assess because of the heterogeneity of the mixed tRNAs.

Translation of VE274hp

The design of the VE274hp construct differs from most naturally-occurring mRNAs in three ways: the existence of internal Shine-Dalgarno (SD)-like sequences, the long Watson-Crick base-paired hairpin (274 bp), and the recurrence of long hydrophobic (15 valines) and short charged (2–5 glutamic acids) amino acid patches in the synthesized protein. Thus, the heterogeneity of translation shown in Fig. 3a can be explained, in part, by these factors.

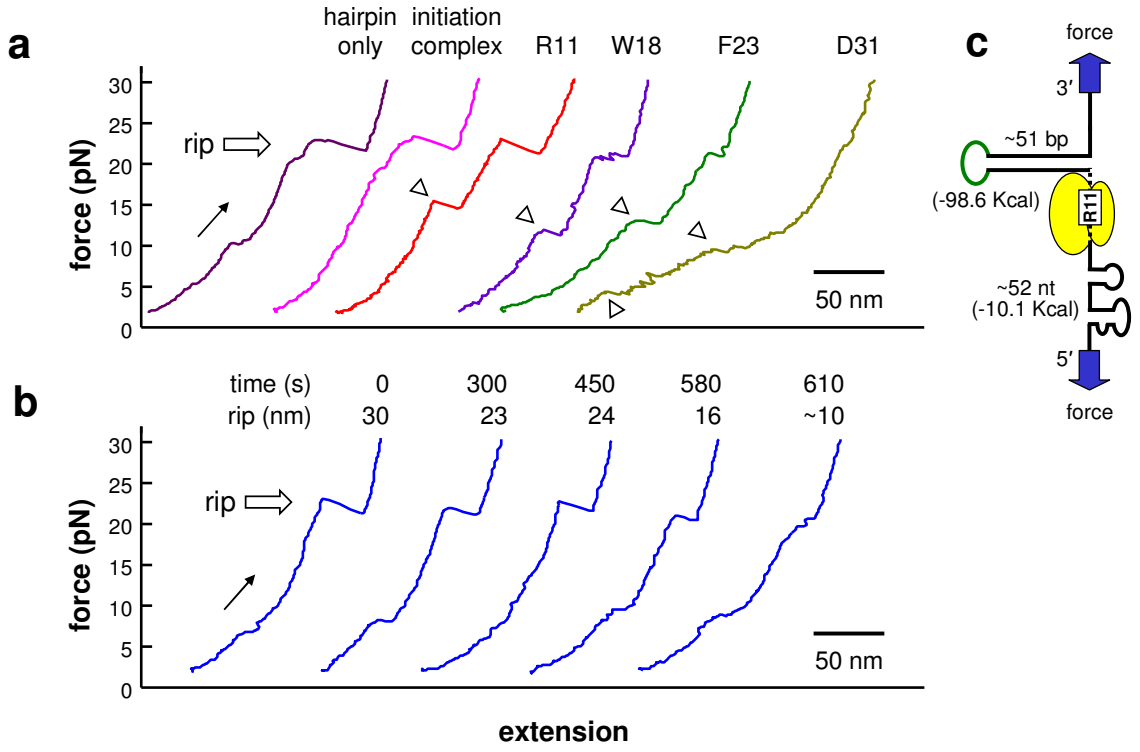
By mutating the sequence, we demonstrated that internal SD-like sequences can cause translation arrests, which accounted for one third of the arrested translation reactions (Fig. 3c). The energy barrier produced by the long hairpin slows down translation, and can also result in longer pauses (up to 2 min). However, the barrier can be reduced by increasing force on the hairpin (Fig. 4b). This is well demonstrated by force-rescuing experiments (Fig. 4c), in which about one third of long translation pauses

resumed elongation when the force acting on the ends of the hairpin was increased. The occurrence of alternating hydrophobic and charged patches in the nascent polypeptide could play a role in slowing translation due to its interaction with the ribosomal exit tunnel. The long hydrophobic valine patch may clog the path of the tunnel by aggregation or interaction with the wall, resulting in long pauses or even arrests. This is supported by the observation that long pauses and translation arrests are not often detected while the first valine patch (V1) is being translated (see Fig. 3c and Supplementary Fig. S6a), and only a few valines have been synthesized.

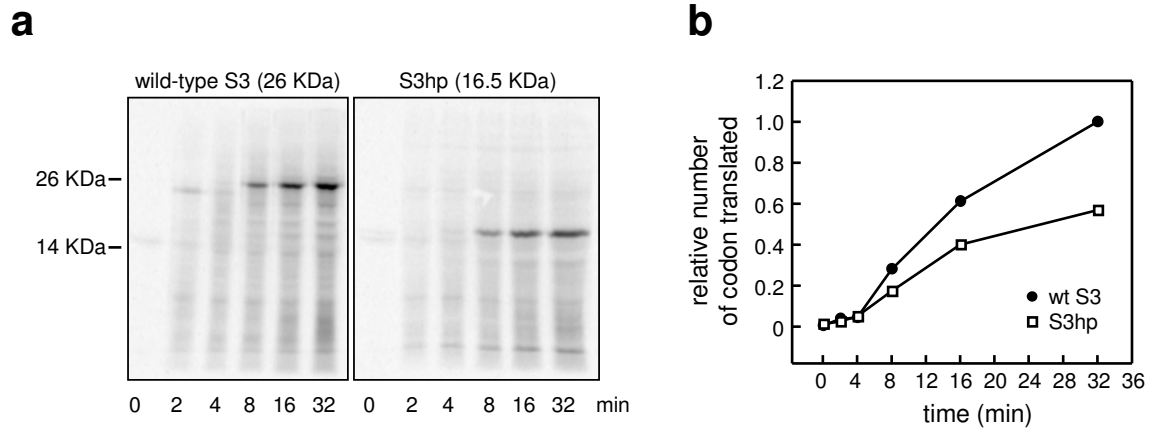
Measurements of translocation times

As mentioned in the main text, the dual-trap tweezers system is excellent in providing low-drift measurements that improve data collection for translation on long templates such as VE274hp, so the dwell times at each codon can be accurately measured over time. However, we also noted that the high-frequency fluctuations were in general bigger; the translation trajectories have to be averaged to ~ 10 Hz to clearly reveal translocation steps, as shown in Fig. 3b. On the other hand, the single-trap tweezers system (with a micropipette, see Fig. 1, left panel) provided less high-frequency fluctuation (< 0.7 nm at 200 Hz; blue lines in Fig. 2a,c), which is significantly smaller than the average translocation distance (~ 2.7 nm). Since the translocation occurs in the timescale of 0.1 s, the stochastic drifts in the system (< 1 nm/s, i.e., up to 0.1 nm in 0.1 s) did not affect the identification of translocation steps. Thus, we used the data measured from the single-trap tweezers for measurement of translocation times, with time and spatial resolutions of 5 ms (at 200 Hz) and 0.7 nm (fluctuations), respectively.

Translocation times were measured at three different constant forces on different ribosomes for the VE60hp mRNA. The mean translocation times were 0.079 ± 0.045 s (n = 35) at 20 pN, 0.076 ± 0.043 s (n = 64) at 18 pN, and 0.079 ± 0.050 s (n = 22) at 15 pN. The distributions of translocation times (data not shown) for each force are similar to Fig. 2d; all distributions are well fit by three substeps of identical rate constants.



Supplementary Figure S1 | Translation snapshots of S3hp. a, Typical force-extension curves of S3hp with stalled ribosomes at designated positions on the hairpin. The stalled positions (in amino acid sequence; see Fig. 1a) are shown above each curve. All traces were from different experiments and different molecules. The unfolding forces and rip sizes for stalled complexes are: 23.1 ± 0.2 pN and 33.8 ± 1.4 nm for R11 (n = 122 traces, from 8 complexes); 21.3 ± 0.7 pN and 22.3 ± 1.7 nm for W18 (n = 278 traces, from 17 complexes); 20 – 21 pN and ~10 nm for F23 (shown are the ranges of multiple transitions, n = 94 traces, from 4 complexes). Note that the rip size gradually decreased as the ribosome was stalled closer to the apical loop of the hairpin. **b,** Force-extension curves of S3hp obtained from the same molecule at different incubation times after adding the translation mixture. The time after injection and the measured rip sizes are shown. For clarity, only the pulling curves are shown in both panels. Open arrows indicate the rips corresponding to unwinding of the hairpins. **c,** Schematic diagram of a representative stalled complex. The ribosome is stalled at R11, resulting in reduction of the hairpin size (~51 bp left). Prediction from *mfold* shows that the free ~52 nt on the 5′ side of the RNA (between the ribosome and the duplex handle) form one of the several branched stem-loop structures with low stability (free energy around -10 Kcal/mol). Those structures are normally opened at lower forces, resulting in rips below 15 pN (indicated by open triangles in panel **a**). Similar results were observed for other stalled complexes.

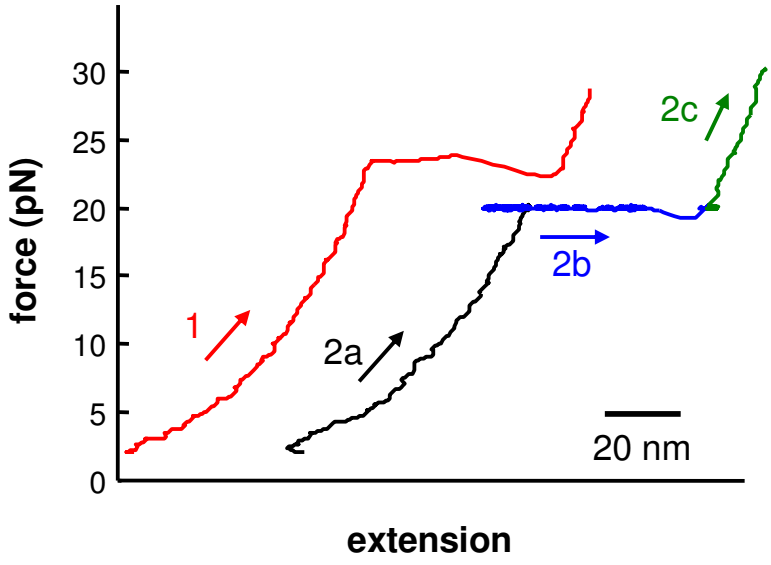


Supplementary Figure S2 | In vitro translation in bulk. In vitro translation was carried out as described previously (see below for reference). Briefly, pre-formed initiation complexes containing 10 pmol of ribosomes, 15 pmol [^{35}S]-fMet-tRNA^{fMet}, 20 pmol mRNA, and 30 pmol each of IF1, IF2, and IF3 in a total volume of 20 µl was added to 80 µl of a reaction mixture containing total aminoacylated-tRNA, [^{35}S]-Met-tRNA^{Met}, 0.05 mM total amino acids minus methionine, 5 mM phosphoenolpyruvate, 1 mM ATP, 1 mM GTP, 2 µl of DEAE purified S-100 enzymes, 80 mM HEPES-KOH (pH7.5), 60 mM NH_4Cl, 10 mM $Mg(OAc)_2$, and 1 mM DTT. The mixture was incubated at 37°C and 15 µl aliquots were quenched at indicated times. The [^{35}S]-labeled translation products were resolved by electrophoresis through a 15% polyacrylamide SDS gel containing 6 M urea and visualized using a Phosphorimager (Molecular Dynamics). **a,** Wild-type S3 mRNA (left, 26 KDa protein) and S3hp mRNA (right, 16.5 KDa protein). The bands show that full-length products are synthesized from each mRNA. **b,** Relative number of codons translated as a function of time. Note that the rate of translation of the wild-type, single-stranded RNA is significantly faster than the hairpin RNA. The quantification (using Imagequant software) has been normalized by the number of [^{35}S]-labeled methionines (4 and 3 for the wild-type S3 and S3hp, respectively) and the total number of amino acids (232 and 150, respectively) in each molecule.

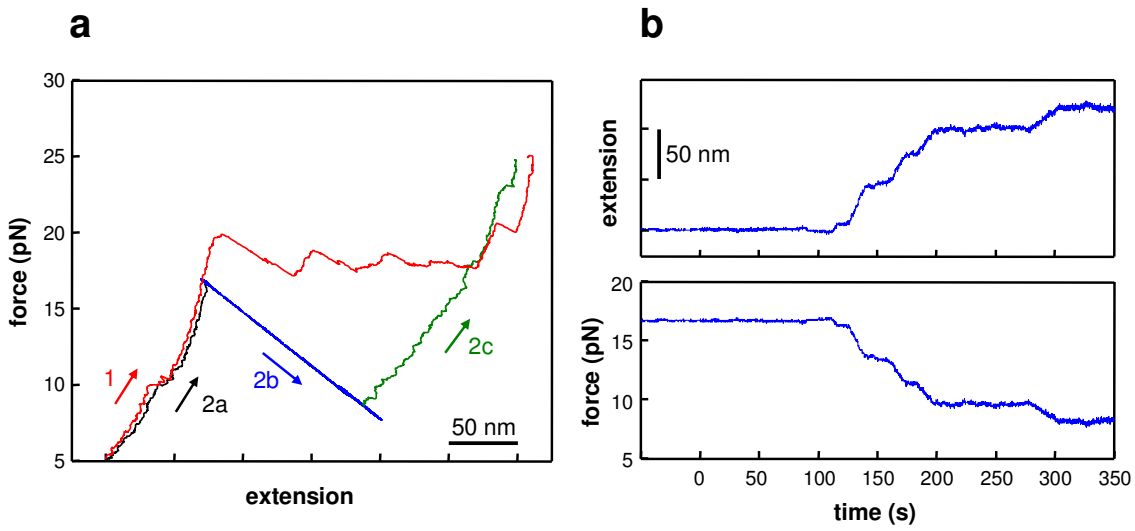
In vitro translation was done for VE60hp and VE274hp mRNAs and compared with their single-stranded versions. Although proteins were synthesized from both the double strands and single strands, aggregation of the polypeptides (even in 6 M urea) produced products with molecular weights much higher than expected. Because of aggregation the sizes of the polypeptides from the different mRNAs could not be determined.

Reference:

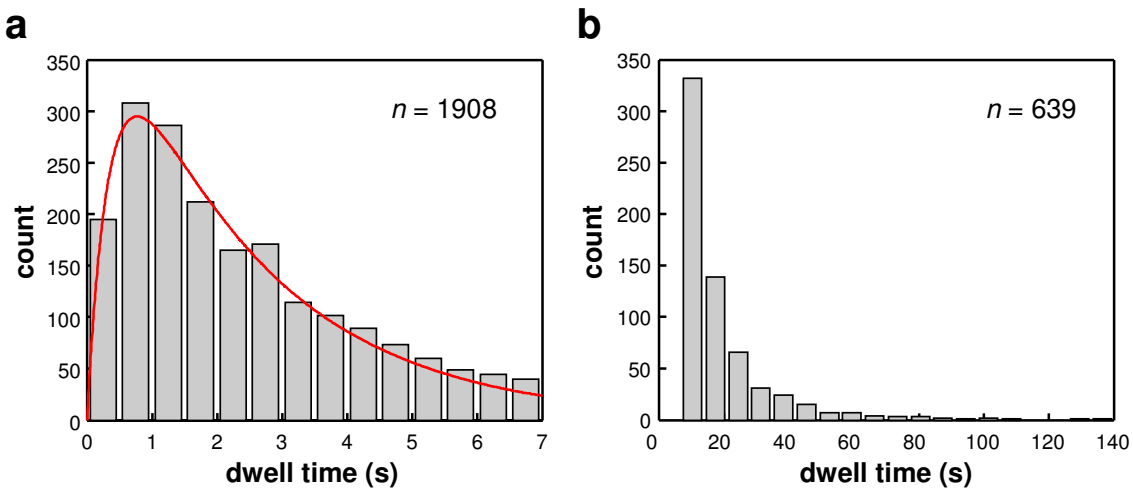
Ali, I. K., Lancaster, L., Feinberg, J., Joseph, S. & Noller, H. F. Deletion of a conserved, central ribosomal intersubunit RNA bridge. *Mol. Cell* **23**, 865-74 (2006).



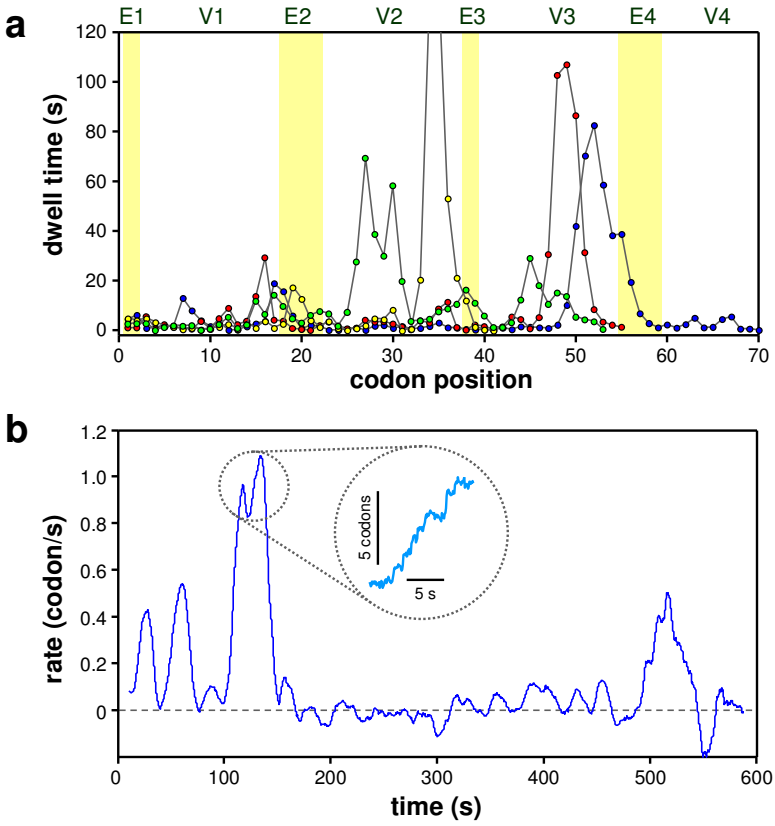
Supplementary Figure S3 | Force-extension curves of VE60hp. The ribosome was initially stalled at a phenylalanine codon (F11, see Fig. 1b) at the base of the hairpin. The construct was pulled (curve 1) and relaxed to confirm that a properly stalled complex was attached, and then pulled again to 20 pN where it was held constant (curve 2a). This force is below that required to unfold the hairpin (~ 23 pN, see curve 1). After injecting the translation mixture, the hairpin opened gradually as the translation proceeded (curve 2b). At the end of the experiment, the construct was pulled (curve 2c) and relaxed to confirm that the ribosome had indeed moved through the hairpin. The extrusion of the translation trace (curve 2b) to the left was caused by drift during sample injection. The two traces were shifted relative to each other for clarity.



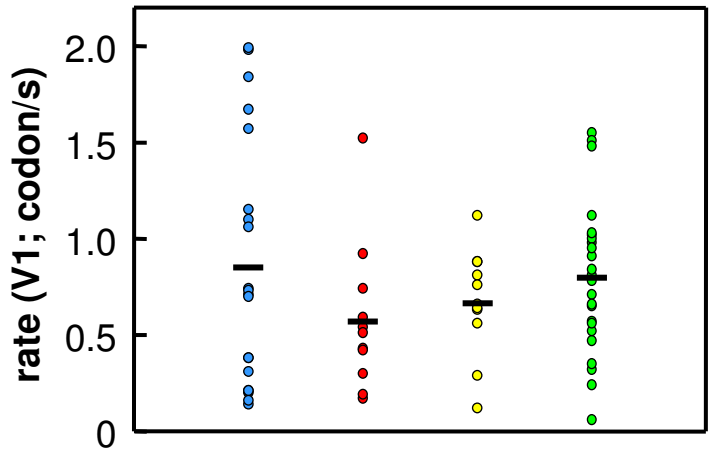
Supplementary Figure S4 | An example of translation using the dual-trap optical tweezers. a, Force-extension curves of VE274hp at various states. The ribosome was initially stalled at the first Glu codon in the hairpin (E20, see Fig. 1c). The construct was pulled (curve 1) and relaxed on the dual-trap tweezers to confirm that a properly stalled complex was tethered. The stalled complex shows five rips on the force-extension curves because of the long hairpin. Note that, due to slight variation in bead size and shape, the force measurements differed by ~ 5% in this case; the unfolding force at the first rip was 22.5 +/- 1.2 pN (n = 39). The construct was pulled to 17 – 18 pN (curve 2a) before flowing in the translation mixture. The hairpin opened gradually by the ribosome as the translation proceeded (curve 2b), as indicated by the blue arrow. Since the trap positions were fixed (no feedback) during the reaction, the force decreased as the extension (end-to-end distance) of the tether increased. At the end of the experiment, the construct was pulled (curve 2c) and relaxed. No apparent rips were detected in the final pulling curve, indicating that the hairpin was already or almost completely opened by the ribosome. **b,** The respective extension (top) and force (bottom) trajectories during translation (corresponding to the blue curve in panel **a**).



Supplementary Figure S5 | Distribution of dwell times. The dwell times from all reaction conditions for VE274hp and VE274hp-G2A (see Supplementary Fig. S7) are separated into two groups with short (< 7 s, panel **a**) and long (> 7 s, panel **b**) durations. We set the boundary between these groups at 7 s such that the mean value for the group with short dwell times (panel **a**) are 2.3 s, close to the intrinsic translation dwell time (2.2 s; see Supplementary Fig. S6b). **a,** Dwell times less than 7 s. n = 1908 (out of 2547), binned in 0.5 s. The distribution best fits a mechanism with two rate-determining kinetic steps (see Supplementary Discussion), with $k_1 = 0.43 \pm 0.05$ s^{-1}, $k_2 = 2.9 \pm 0.7$ s^{-1}, and $R^2 = 0.98$. **b,** Dwell times larger than 7 s. n = 639, binned in 7 s intervals. The mean and median values are 19 and 13 s, respectively.



Supplementary Figure S6 | Long pauses during translation. Individual translation trajectories for VE274hp were quantitatively analyzed. **a,** Plots of dwell times at each codon. Shown are four examples in different colors. Valine (V1 – V4) and glutamic acid (E1 – E4, yellow bars) patches are indicated. Note that long pauses are less often observed in V1. **b,** Translation rates as a function of time. The trace is taken from the same data as the blue trace in the top panel. The rates are calculated by taking the first derivatives (using a window of 20 s) of the translation trajectory (as shown in Fig. 3a; 10 Hz bandwidth). There are four major peaks in this example. The valleys between peaks are longer pauses that can be affected by the factors as discussed in the Supplementary Discussion. The translation in the peak regions (as shown in the inset) represents constitutive (intrinsic) translation, which is less affected by the factors causing the long pauses. The average rate in the peak regions is 0.45 ± 0.17 codon/s (n = 43 ribosomes), corresponding to a mean dwell time of 2.2 s. Note that the long pause phase between 150 – 480 s corresponds to translation near codon #52 (top panel, blue circles). The ribosome hardly moved during this period, but translocated rapidly at time 500 s. This could be interpreted as, for example, clearance of the hydrophobic peptide clogging the ribosomal exit tunnel (see Supplementary Discussion).



VE274hp	wt	wt	wt	G2A
EF-G (μM)	1	0.1	1	1
Val-tRNA^{Val} (μM)	0.7	0.7	0.07	0.7
n	20	11	11	24

Supplementary Figure S7 | Translation rates of VE274hp. The translation reaction was done under four different conditions with changes in the concentrations of EF-G, Val-tRNA^{Val}, or sequences of hairpins (wt refers to the wild-type VE274hp, and G2A refers to the mutant VE274hp-G2A). The rates shown here are for the first Val patch (V1) only. The average value for each condition is shown with a horizontal bar.