

Supporting Information

Inefficient delivery but fast peptide bond formation of unnatural L-aminoacyl-tRNAs in translation

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Comparison of published measurements of dissociation (equilibrium) constant (K_d) for binding of Phe-tRNA^{Phe} to EF-Tu:GTP

13 nM¹ and 27 nM² (heterologous *T. thermophilus* EF-Tu and *E. coli* Phe-tRNA^{Phe} at 4 °C);
0.6 nM^{3,4} (*E. coli* EF-Tu and *E. coli* Phe-tRNA^{Phe} at 5 °C);
0.02 μM (heterologous *E. coli* EF-Tu and yeast Phe-tRNA^{Phe} at 25 °C)⁵;
0.08 μM⁵ (heterologous *E. coli* EF-Tu and yeast Phe-tRNA^{Phe} at 37 °C);
0.002 μM³ and 0.07 μM⁶ (*E. coli* EF-Tu and *E. coli* Phe-tRNA^{Phe} at 37 °C).

As the equilibrium constant is sensitive to buffer², temperature^{2,3,5} and pH, our measurements may differ from published data due to different experimental conditions. There was no significant difference for binding to the heterologous *T. Thermophilus* EF-Tu between modified and unmodified AA-tRNAs².

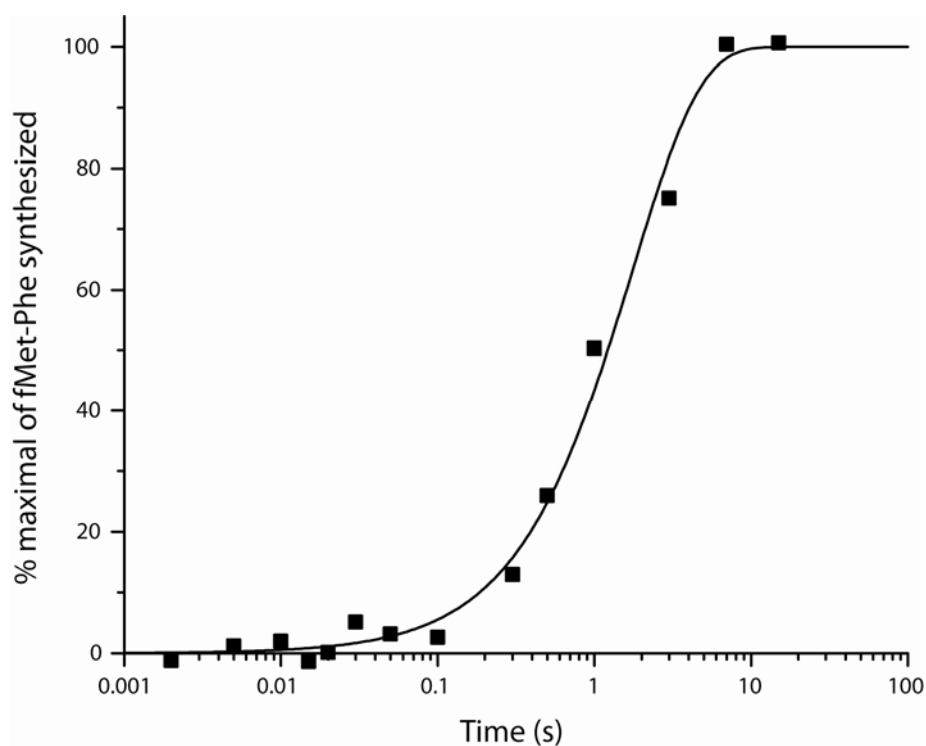


Figure S1. Kinetics of dipeptide (fMet-Phe) synthesis from fMet-tRNA_i^{fMet} and natural Phe-tRNA^{Phe} in the absence of pre-formed ternary complexes. The dipeptide formation was started by adding Phe-tRNA^{Phe} to the ribosome mixture containing 10μM EF-Tu (see Experimental Section in the main text for composition of the mixtures. The ternary complex mixture contained no EF-Tu in this case.) The data were fitted to a single step exponential model and the rate was estimated to be $0.57 \pm 0.053 \text{ s}^{-1}$.

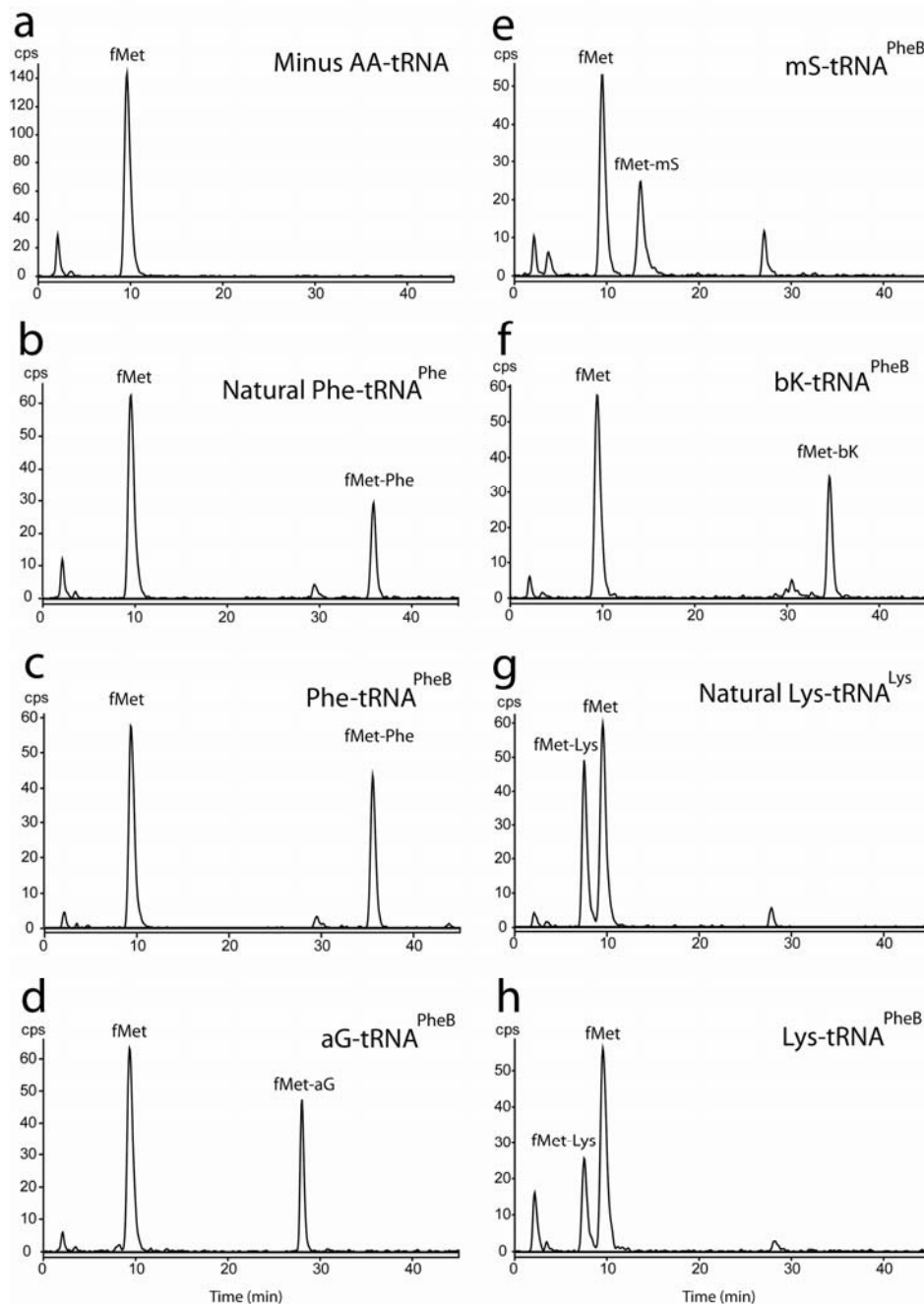


Figure S2. Reversed-phase HPLC elution profiles of dipeptide products from $[^3\text{H}]\text{fMet-tRNA}_i^{\text{fMet}}$ and natural $\text{Phe-tRNA}^{\text{Phe}}$ (b), natural $\text{Lys-tRNA}^{\text{Lys}}$ (g) or $\text{tRNA}^{\text{PheB}}$ charged with Phe (c), aG (d), mS (e), bK (f) or Lys (h). A control experiment (a) was done without ternary complexes added. Dipeptide synthesis reactions were carried out for 10 s (except $\text{bK-tRNA}^{\text{PheB}}$ (f) for 200 s) at 37°C , and samples for HPLC analysis were prepared as described in Experimental Section in the main text. Counts per second (cps) were plotted against elution time (min). (a–h) Elution for 0–20 min was isocratic with 0.1% trifluoroacetic acid/7.2% methanol and elution for 20–45 min was at 0.1% trifluoroacetic acid/36% methanol. Elution profile of dipeptide product from $[^3\text{H}]\text{fMet-tRNA}_i^{\text{fMet}}$ and unmodified $\text{Phe-tRNA}^{\text{Phe}}$ was identical to (b) and (c). Natural $\text{Lys-tRNA}^{\text{Lys}}$ was prepared by charging *E. coli* tRNA^{Lys} with natural Lys by *E. coli* LysRS.

Supporting References

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- (2) LaRiviere, F. J.; Wolfson, A. D.; Uhlenbeck, O. C. *Science* **2001**, *294*, 165.
- (3) Ott, G.; Faulhammer, H. G.; Sprinzl, M. *Eur J Biochem* **1989**, *184*, 345.
- (4) Ott, G.; Schiesswohl, M.; Kieseewetter, S.; Forster, C.; Arnold, L.; Erdmann, V. A.; Sprinzl, M. *Biochim Biophys Acta* **1990**, *1050*, 222.
- (5) Pingoud, A.; Urbanke, C.; Krauss, G.; Peters, F.; Maass, G. *Eur J Biochem* **1977**, *78*, 403.
- (6) Tapio, S.; Bilgin, N.; Ehrenberg, M. *Eur J Biochem* **1990**, *188*, 347.

Theoretical analysis of experimental data

The simplest explanation for the biphasic kinetics of dipeptide formation observed in our experiments comes from the kinetic scheme (1) describing a one-step formation of ternary complex (T3) from an aminoacylated tRNA (aa-tRNA) and EF-Tu (Tu):

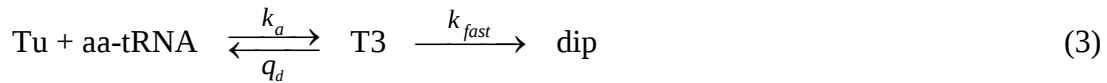


We will assume here that Tu is in its GTP form competent in aa-tRNA binding. We will also assume that Tu is in large excess over aa-tRNA, the condition satisfied for the majority of experiments in this study. With these assumptions, the T3 concentration in the T3 mixture (see Experimental Section for the composition of the T3 mixture) before the addition of ribosomal complexes (RC) is:

$$[\text{T3}] = [\text{aa-tRNA}] \cdot [\text{Tu}] / ([\text{Tu}] + K_d) \quad (2)$$

Here, $K_d = q_d / k_a$ is the dissociation equilibrium constant of T3 formation and $[\text{aa-tRNA}]$ is the total aa-tRNA concentration in the T3 mixture, so that the ratio $[\text{Tu}] / ([\text{Tu}] + K_d)$ gives the fraction of total aa-tRNA in the ternary complex with Tu:GTP.

When the T3 mixture and the RC mixture (see Experimental Section for the composition of the RC mixture) are mixed together in the quench flow apparatus, the formation of dipeptide (dip) occurs according to the kinetic scheme (3):



Under our experimental conditions when we have a large excess of ribosome complexes over available ternary complexes, the rate of dipeptide formation, k_{fast} in the scheme (3), depends on the concentration of RC and the standard Michaelis Menten parameters k_{cat} and K_{T3} of the dipeptide formation reaction according to:

$$k_{fast} = k_{cat} \cdot [\text{RC}] / ([\text{RC}] + K_{T3}) \quad (4)$$

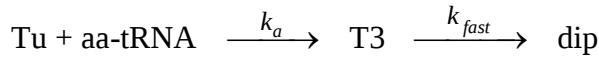
Scheme (3) implies that the T3 complexes formed during the pre-incubation of T3 mixture in the absence of RC react fast with the added RC and become rapidly consumed with the rate k_{fast} in the fast phase of dipeptide formation as observed experimentally (e.g. Figure 2a in the main text).

In parallel with the consumption of the pre-formed T3, another slower process will also take place. In this slow process, the remaining free aa-tRNAs bind to Tu with the effective association rate $k_{ass} = k_a \cdot [\text{Tu}]$ forming new ternary complexes T3 that either dissociate with the rate q_d or become consumed in the dipeptide formation reaction with the rate k_{fast} . It can be shown (see Appendix) that in

the case when k_{fast} is much faster than the equilibration in the reaction (1) (i.e. $k_{fast} \gg k_a [Tu] + q_d$), the effective rate of this slow process k_{slow} is approximated by:

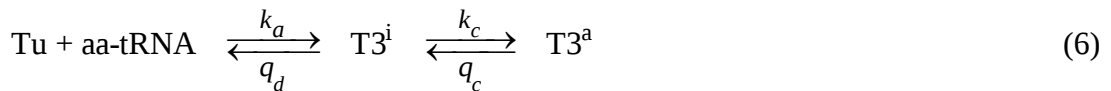
$$k_{slow} \approx k_a [Tu] \frac{1}{(1 + (k_a [Tu] + q_d) / k_{fast})} \approx k_a [Tu] \quad (5)$$

The approximation $k_{slow} \approx k_a [Tu]$ follows also directly from the kinetic scheme (3) if one notice that since $k_{fast} \gg q_d$, the probability for the formed T3 to dissociate back into free Tu and aa-tRNA instead of interacting with the ribosome is close to zero. It means that for dipeptide formation one can neglect q_d in the kinetic scheme (3) and just consider the simplified kinetic scheme:



For this simplified scheme the rate of the slow phase is equal exactly to $k_a [Tu]$ (i.e. $k_{slow} = k_a [Tu]$).

Relation (5) shows that the rate of the slow phase of dipeptide formation, k_{slow} , should increase almost linearly with the increase in Tu concentration. However, contrary to this prediction, our experimental observations compiled in Table 1 show only modest variations in k_{slow} in a wide range of Tu concentrations. In addition, Eq (2) derived from the kinetic scheme (1) predicts the increase of the fast phase to 100% at sufficiently high Tu concentration. In contrast, our experiments show that the fast phase of di-peptide formation never exceeded 90% even at saturating Tu concentrations. These two major deviations from the experimental observation prompted us to postulate the existence of two forms of T3, one active ($T3^a$) and another inactive ($T3^i$) in dipeptide formation. We then modified the simple kinetic models (1) to include these two forms of T3 as shown below:



Similarly to relation (2), the total concentration of T3 for the scheme (6) is given by:

$$[T3] = [T3^i] + [T3^a] = [aa-tRNA][Tu] / ([Tu] + K_d) \quad (7)$$

However, in this case, the equilibrium dissociation constant K_d is related to the elemental rate constants in the scheme (6) by:

$$K_d = (q_d / k_a) / (1 + k_c / q_c) \quad (8)$$

The fraction of inactive T3 in total T3 is given by:

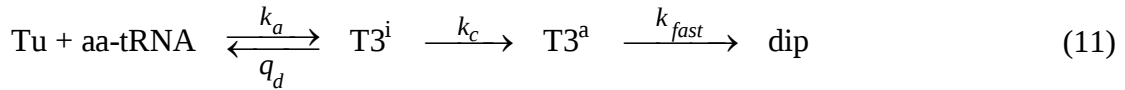
$$\frac{[T3^i]}{[T3]} = q_c / (k_c + q_c) \quad (9)$$

This relation shows that the fraction of inactive T3 in total T3 depends only on the rates of slow conformational transitions relating these two forms but not on the Tu concentration in the T3 mixture. The corresponding modified kinetic scheme for dipeptide formation (which starts after the RC addition) is:



This scheme implies that after RC addition all active T3 complexes that have been formed during pre-incubation of the T3 complex mixture in the absence of RC will be consumed with the rate close to k_{fast} in dipeptide formation reaction giving rise to the fast phase observed experimentally. In parallel, a slow process which has two origins will take place. Firstly, at high Tu concentrations the slow phase originates mainly from pre-formed inactive ternary complexes that have to switch to active form before they can participate in dipeptide formation. The rate of dipeptide formation reaction starting from inactive T3 is then limited by the rate, k_c , of the inactive T3 to active T3 conversion. Secondly, at intermediate Tu concentrations when a considerable fraction of aa-tRNA is free, the slow process would also include aa-tRNA binding to Tu and the formation of inactive T3.

To obtain an approximation for the rate of the slow process we can again invoke the probability argument used above. Namely, the active T3 formed from inactive T3 in the scheme (10) has almost zero probability to transform back into inactive T3 since we have assumed here that transitions between active and inactive T3 forms are slow in comparison with the rate of dipeptide formation (i.e. $k_{fast} \gg q_c$). We can then neglect q_c in the scheme (10) and consider a simplified scheme of dipeptide formation:



From scheme (11) the approximation for the rate k_{slow} of the slow process that includes aa-tRNA binding to Tu, formation of inactive T3, its subsequent activation and dipeptide formation is given by (see Appendix):

$$k_{slow} \approx k_c \frac{[Tu]}{([Tu] + (k_c + q_d) / k_a)} \approx k_c$$

This relation shows that if the Tu concentration is higher than $K_f \approx (k_c + q_d) / k_a$, then the increase in Tu concentration would have only a small effect on k_{slow} . The value of K_f is of the order of K_d meaning

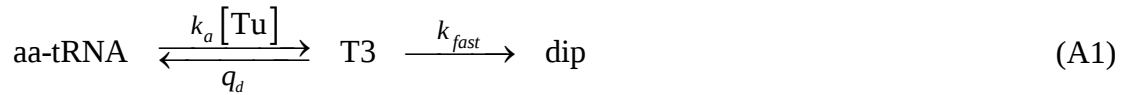
that at Tu concentrations above K_d only a small increase in k_{slow} is to be expected, as we generally observe. On the other hand, when the Tu concentration is much below K_d one would expect a close to linear dependence between k_{slow} and the Tu concentration that we indeed observed in the case of bK where the slow phase was much slower than for the other unnatural AAs (see Table 1 in the main text).

Further, relation (9) shows that even at saturating Tu concentrations, when all aa-tRNA is chased into T3 complexes, the fraction of inactive T3 in total T3 remains constant and equal to the $q_c / (k_c + q_c)$ ratio explaining why the slow phase cannot be eliminated even at high Tu concentration. For example, the persistence of the slow phase of 20% amplitude in our experiments even at saturating Tu concentrations can be readily explained assuming that $q_c = k_c / 4$.

Finally, the K_d values can be easily found plotting a suitably normalized fraction of the fast phase versus Tu concentration in the T3 mixture as explained in Appendix (see below).

Appendix

Reaction scheme (3) can be presented in the equivalent form:



The kinetics of the aa-tRNA disappearance and kinetics of T3 formation and disappearance can be described by the equation system in the matrix form as:

$$\frac{d}{dt} \begin{bmatrix} \text{aa-tRNA} \\ \text{T3} \end{bmatrix} = \begin{bmatrix} -k_a [\text{Tu}] & q_d \\ k_a [\text{Tu}] & -q_d - k_{fast} \end{bmatrix} \begin{bmatrix} \text{aa-tRNA} \\ \text{T3} \end{bmatrix} \quad (\text{A2})$$

Eigen values, p-s, of the matrix of the equation system give the rates in the exponential terms that describe the aa-tRNA and T3 disappearance. These eigen values are obtained as the solutions of the eigen equation:

$$\det \begin{bmatrix} -k_a [\text{Tu}] - p & q_d \\ k_a [\text{Tu}] & -q_d - k_{fast} - p \end{bmatrix} = 0 \quad (\text{A3})$$

Or:

$$p^2 + (k_a [\text{Tu}] + q_d + k_{fast})p + k_a [\text{Tu}] \cdot k_{fast} = 0 \quad (\text{A4})$$

The solutions for p are:

$$p_{1,2} = -\frac{k_a [\text{Tu}] + q_d + k_{fast}}{2} \pm \frac{k_a [\text{Tu}] + q_d + k_{fast}}{2} \sqrt{1 - \frac{4k_a [\text{Tu}] \cdot k_{fast}}{(k_a [\text{Tu}] + q_d + k_{fast})^2}} \quad (\text{A5})$$

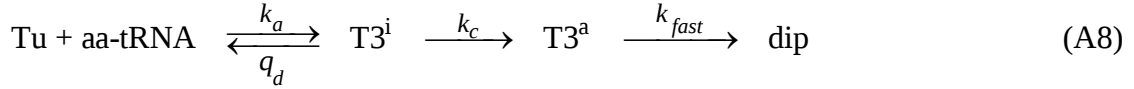
Assuming that $k_{fast} \gg k_a [\text{Tu}] + q_d$ one can use a Taylor expansion of the square root in (A5) to get:

$$p_{1,2} \approx -\frac{k_a [\text{Tu}] + q_d + k_{fast}}{2} \pm \frac{k_a [\text{Tu}] + q_d + k_{fast}}{2} \left\{ 1 - \frac{2k_a [\text{Tu}] \cdot k_{fast}}{(k_a [\text{Tu}] + q_d + k_{fast})^2} \right\} \quad (\text{A6})$$

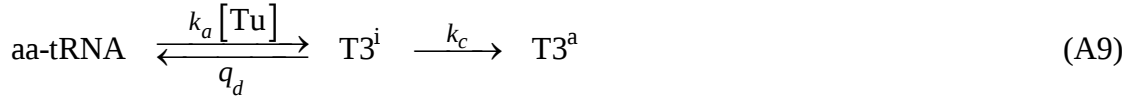
For the slow rate that corresponds to the smallest eigen value one gets:

$$k_{slow} = -p_1 \approx \frac{k_a [\text{Tu}] \cdot k_{fast}}{(k_a [\text{Tu}] + q_d + k_{fast})} = k_a [\text{Tu}] \frac{1}{(1 + (k_a [\text{Tu}] + q_d) / k_{fast})} \quad (\text{A7})$$

We now derive the relation for the slow rate for the reaction scheme:



We first notice that the kinetics of free aa-tRNA disappearance and the kinetics of the formation and disappearance of inactive T3 in the scheme (A8) determine completely the rate of the slow process. These kinetics are described by a two-step scheme:



We can then essentially repeat the exposition in the discussion of the kinetic scheme (A1) above to arrive at the expression:

$$p_{1,2} = -\frac{k_a [\text{Tu}] + q_d + k_c}{2} \pm \frac{k_a [\text{Tu}] + q_d + k_c}{2} \sqrt{1 - \frac{4k_a [\text{Tu}] \cdot k_c}{(k_a [\text{Tu}] + q_d + k_c)^2}} \quad (\text{A10})$$

For the slow rate that corresponds to the smallest eigen value one then gets:

$$k_{slow} = -p_1 \approx \frac{k_a [\text{Tu}] \cdot k_c}{(k_a [\text{Tu}] + q_d + k_c)} = k_c \frac{[\text{Tu}]}{([\text{Tu}] + (q_d + k_c) / k_a)} \quad (\text{A11})$$

The approximation (A11) is valid under the assumption $k_c \ll k_a [\text{Tu}] + q_d$.

Strictly speaking, the expression for the time evolution of dipeptide formation for the three step kinetic scheme (A8) should include three exponential terms with the rates k_{slow} , k_{fast} and also with the intermediate rate k_{in} corresponding to the $-p_2$ eigen value in expression (A10). The approximation for this rate is:

$$k_{in} = -p_2 \approx k_a [Tu] + q_d + k_c \frac{(q_d + k_c) / k_a}{([Tu] + (q_d + k_c) / k_a)} \quad (A12)$$

However, under the condition $k_c \ll k_a [Tu] + q_d$ we will also have $k_{slow} < k_c \ll k_{in}$ which implies a negligible amplitude of the process with the rate k_{in} . Indeed, the exact solution for the time course of dipeptide formation according to the scheme (A8) can be written as:

$$\begin{aligned} \text{dip}(t) = & \frac{([aa\text{-tRNA}(0)] - [T3^a(0)]) - [T3^i(0)] k_c / k_{in}}{(1 - k_{slow} / k_{in})(1 - k_{slow} / k_{fast})} \cdot Ux(-k_{slow}t) \\ & - \frac{k_{slow}}{k_{in}} \cdot \frac{([aa\text{-tRNA}(0)] - [T3^a(0)]) - [T3^i(0)] k_c / k_{slow}}{(1 - k_{slow} / k_{in})(1 - k_{in} / k_{fast})} \cdot Ux(-k_{in}t) \\ & + \left\{ [T3^a(0)] + \frac{([aa\text{-tRNA}(0)] - [T3^a(0)]) k_{in} k_{slow} / k_{fast}^2 - [T3^i(0)] k_c / k_{fast}}{(1 - k_{slow} / k_{fast})(1 - k_{in} / k_{fast})} \right\} \cdot Ux(-k_{fast}t) \end{aligned} \quad (A13)$$

Here, $[aa\text{-tRNA}(0)]$, $[T3^a(0)]$ and $[T3^i(0)]$ correspond to the total concentration of aa-tRNA, active and inactive forms of T3 before the addition of ribosome complexes. We have also introduced the exponential terms in the form: $Ux(-k \cdot t) \equiv 1 - \exp(-k \cdot t)$.

Using the conditions $k_{slow} < k_c \ll k_{in} < k_{fast}$ we can neglect terms in (A13) multiplied by k_{slow} / k_{in} or k_c / k_{in} and by k_{slow} / k_{fast} or k_c / k_{fast} to approximate the complex expression (A13) by a simple formula:

$$\text{dip}(t) \approx ([aa\text{-tRNA}(0)] - [T3^a(0)]) \cdot (1 - \exp(-k_{slow}t)) + [T3^a(0)] \cdot (1 - \exp(-k_{fast}t)) \quad (A14)$$

Relation (A14) shows directly that the amplitude of the fast exponential term is equal to the concentration of active T3 before the start of dipeptide formation reaction. In addition, as expected, the total amplitude of the dipeptide formation reaction is equal the total initial aa-tRNA concentration. Further, the fractional amplitude of the fast phase corresponds to the ratio $[T3^a(0)] / [aa\text{-tRNA}(0)]$ in the T3 mixture before the start of dipeptide formation.

We can use relations (7) and (9) in the main text to write:

$$P_{\text{fast}} = \left[\text{T3}^{\text{a}} \right] / \left[\text{aa-tRNA} \right] = \frac{k_c}{k_c + q_c} \frac{[\text{Tu}]}{[\text{Tu}] + K_d} = P_{\text{fast, max}} \frac{[\text{Tu}]}{[\text{Tu}] + K_d} \quad (\text{A15})$$

The relation (A15) shows that the fit of the Tu-dependence of the fast phase fraction (P_{fast} , which is estimated from our experimental data, and is equal to $\left[\text{T3}^{\text{a}}(0) \right] / \left[\text{aa-tRNA}(0) \right]$ ratio) with a two-parameter hyperbolic function would give us both the value of K_d and the value of $P_{\text{fast, max}} = k_c / (k_c + q_c)$. Alternatively, one can re-normalized the amplitude of the fast phase by the amplitude of the fast phase at saturating Tu concentrations ($P_{\text{fast, max}}$, as it was done in this study). Clearly, the values of the K_d parameter obtained from a hyperbolic fit do not depend on the particular way of normalization of the amplitude of the fast phase.