pH-sensitivity of the ribosomal peptidyl transfer reaction dependent on the identity of the A-site aminoacyl-tRNA

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We studied the pH-dependence of ribosome catalyzed peptidyl transfer from fMet-tRNAfMet to the aa-tRNAs Phe-tRNAPro, Ala-tRNAAla, Gly-tRNAPro, Pro-tRNAPro, Asn-tRNAAsn, and Ile-tRNAIle, selected to cover a large range of intrinsic pKα-values for the α-amino group of their amino acids. The peptidyl transfer rates were different at pH 7.5 and displayed different pH-dependence, quantified as the pH-value, pKαobs, at which the rate was half maximal. The pKαobs-values were downshifted relative to the intrinsic pKα-value of aa-tRNAs in bulk solution. Gly-tRNAPro had the smallest downshift, while Ile-tRNAIle and Ala-tRNAAla had the largest downshifts. These downshifts correlate strongly with molecular dynamics (MD) estimates of the downshifts in pKα-values of these aa-tRNAs upon A-site binding. Our data show the chemistry of peptide bond formation to be rate limiting for peptidyl transfer at pH 7.5 in the Gly and Pro cases and indicate rate limiting chemistry for all six aa-tRNAs.

The ribosome promotes protein elongation by transfer of the nascent peptide chain from P-site peptide-tRNA to A-site aminoacyl-tRNA (aa-tRNA) (Fig. 1) and translocation of messenger RNA (mRNA) and tRNAs. Peptide bond formation is initiated by a nucleophilic attack of the α-amino group of the amino acid, ester linked to the A-site tRNA, on the ester carbonyl carbon of the peptide chain linked to the 3′-oxygen of the P-site tRNA (Fig. 2). Biochemical data (1–3), crystallographic data (4–6), and molecular dynamics simulations (7, 8) have shown that the 2′OH group of A76 of the P-site tRNA greatly accelerates peptide bond formation by providing a shuttle of the proton from the attacking α-amino group to the leaving 3′O of the deacylated P-site tRNA. The rate of ribosomal peptidyl transfer is further accelerated by a network of H-bonds involving water molecules and conserved bases in the peptidyl transferase center (PTC) of the ribosome (4, 8, 9).

Peptidyl transfer requires that the α-amino group of the amino acid on the A-site tRNA is in charge neutral rather than in protonated NH₃⁺ form (Fig. 2) (10). At 25 °C the pKα-values of the α-amino groups of amino acids in bulk water range from 8.8 (Asn) to 10.6 (Pro) units (11). The pKα-values of the aa-tRNAs, approximated by the pKα-values of the corresponding amino acid methyl and ethyl esters (12–14), are downshifted in relation to those of the amino acids by two units, with 20 °C values ranging from 6.8 (Asn) to 8.6 (Pro) (Table 1). The rate of ribosomal peptidyl transfer might therefore be expected to vary differently with pH in the physiological range 6–8 for different A-site bound aa-tRNAs. The sensitivity of peptide bond formation to pH-variation in the 6–8 range has so far only been observed for aa-tRNA analogues but not for native aa-tRNAs (15). It has been suggested that the lack of pH sensitivity for aa-tRNAs is that their accommodation in the A site is so slow that the expected pH-sensitivity of the chemistry of peptidyl transfer is completely masked (16, 17). This view (18–21) was recently challenged in a study (22), based on an Escherichia coli system for protein synthesis optimized for speed and accuracy. Here the activation enthalpy and entropy for peptidyl transfer from initiator tRNA to a native aa-tRNA were similar to the corresponding activation parameters for peptidyl transfer to puromycin, lacking a rate limiting accommodation step (23). From this similarity it was argued that peptidyl transfer to native aa-tRNA and to puromycin may both be rate-limited by the chemistry of peptide bond formation (22). Indeed, incorporation rates in translation of N-alkyl aa-tRNAs (including natural Pro-tRNAs) vs. Phe- and Ala-tRNAs differ in a manner that correlates strongly with α-amino group reactivity (24–26).

Here, we studied the pH-dependence of the rate of peptidyl transfer from P-site bound initiator tRNA to six different aa-tRNAs entering the ribosome in ternary complex with EF-Tu and GTP. The aa-tRNAs were selected to cover a large range of pKα-values for their α-amino groups (Table 1, left). We used the linear interaction energy (LIE) method (27, 28) to estimate the shift between the pKα-values of these aa-tRNAs in bulk water and in A site.

We conclude that the chemistry of peptidyl transfer limits the rate of peptidyl transfer to Gly-tRNAPro and Pro-tRNAPro at physiological pH, and that a high pKα-value of Pro-tRNAPro contributes to its small rate of peptidyl transfer (24). This result shows that the chemistry of peptidyl bond formation and its pH-dependence are relevant for the rate of protein elongation in the living cell and opens a window for kinetic studies of peptide bond formation on the ribosome with native tRNA substrates. The strong correlation that we observe between the molecular dynamics (MD) simulated and the kinetically estimated, apparent pKα-shifts for the six aa-tRNAs suggests that the chemistry of peptide bond formation could, surprisingly, be rate limiting for peptidyl transfer to all six aa-tRNAs at all pH-values.

Results

pH-Dependence of the Rate of Ribosomal Peptidyl Transfer. The pKα-values of the α-amino groups of aa-tRNAs in bulk solution, approximated by the pKα-values of the corresponding methyl and ethyl esters (12–14), vary greatly with the amino acid side groups.


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We have selected six aa-tRNAs (Table 1, column 1) with bulk solution $pK_a$-values in the 6.8 (Asn-tRNA$^{\text{Asn}}$) to 8.6 (Pro-tRNA$^{\text{Pro}}$) pH unit interval (Table 1, column 2). The pH-dependence of the rate of ribosome catalyzed peptidyl transfer from initiator tRNA precedes accommodation, so that $\tau_{\text{pep}}$ includes the time, $1/k_{\text{tu}}$, if release of EF-Tu-GDP from the ribosome occurs in parallel with accommodation (16), then $1/k_{\text{tu}}$ would not be included in $\tau_{\text{pep}}$ (See also Data Evaluation in SI Text).

For measurement precision, GTP hydrolysis and dipeptide formation were always monitored in the ternary complex, consisting of EF-Tu, $[^3\text{H}]$GTP and one of the six aa-tRNAs, was rapidly mixed in a quench-flow instrument with a single isoaccepting tRNA, charged with amino acid in bulk or in partially purified form (tRNAPhe). After mixing, the $[^3\text{H}]$GTP was separated from GTP by TLC or HPLC and fMet from fMet-aa by RP-HPLC.

For measurement precision, GTP hydrolysis and dipeptide formation were always monitored in the very same experiment. The average times for GTP hydrolysis ($\tau_{\text{GTP}}$, see SI Text: Eq S2) and dipeptide formation ($\tau_{\text{dip}}$, see SI Text: Eq S1) and their standard errors were estimated by fitting Eqs. S4 (single exponential kinetics, SI Text) and S5 (double exponential kinetics, SI Text) to experimental data. The average time for peptidyl transfer ($\tau_{\text{pep}}$) was estimated as the difference $\tau_{\text{pep}} = \tau_{\text{dip}} - \tau_{\text{GTP}}$, illustrated schematically in Fig. 1 and graphically as the shaded area in Fig. S4 and Fig. S1 (See Data Evaluation in SI Text). In all cases $\tau_{\text{pep}}$ decreased significantly when pH increased.

We have defined a “compounded” rate constant, $k_{\text{pep}}$, for all steps after GTP hydrolysis up to and including peptide bond formation (see Fig. 1 for details) as the inverse of $\tau_{\text{pep}}$, i.e., $k_{\text{pep}} = 1/\tau_{\text{pep}}$. Values of $k_{\text{pep}}$ estimated from quench-flow data at different pH-values are shown in Figs. 3 B–G.

In the Pro and Gly cases, plots of $\log_{10} k_{\text{pep}}$ vs. pH are linear at low pH with positive slopes close to one (Fig. 3F). At further pH-increase, $\log_{10} k_{\text{pep}}$ asymptotically approaches its maximal value, $\log_{10} k_{\text{pep}}^\text{max}$. For these two aa-tRNAs the variation of $k_{\text{pep}}$ with pH was due to the titration of a reaction-essential group with a single proton. This group, we propose, is the a-amino group of tRNA-bound Gly or Pro in the ribosomal A site (Fig. 2). By inference, we suggest that the variation of $k_{\text{pep}}$ with pH for all six aa-tRNAs (Fig. 3) is due to single proton titration on the a-amino group, i.e., (see SI Text):

$$k_{\text{pep}} = \frac{k_{\text{pep}}^\text{max}}{1 + 10^{(pK_a^\text{obs} - \text{pH})}}.$$  

Here $pK_a^\text{obs}$ is operationally defined as the pH-value at which $k_{\text{pep}} = k_{\text{pep}}^\text{max}/2$. Estimates of $pK_a^\text{obs}$ and $k_{\text{pep}}^\text{max}$ for all six aa-tRNAs are shown in Table 1. The largest $pK_a^\text{obs}$-values are seen for Pro ($pK_a^\text{obs} = 7.8$) and Gly ($pK_a^\text{obs} = 7.4$), and the smallest for Asn ($pK_a^\text{obs} = 5.9$), while intermediate $pK_a^\text{obs}$-values are seen for Phe ($pK_a^\text{obs} = 6.1$), Ile ($pK_a^\text{obs} = 6.1$), and Ala ($pK_a^\text{obs} = 6.3$) (Table 1 and Fig. 3). The $pK_a^\text{obs}$-values are downshifted in relation to the $K_a^\text{obs}$ values for aa-tRNAs in bulk solution, $pK_a^\text{aq}$, by about one unit in the Asn, Phe, and Pro cases, 1.5 unit in the Ile and Ala cases, and 0.4 unit in the Gly case (Table 1, columns 2 and 3).

The $K_a^\text{max}$-values were largest for the fast aa-tRNAs cognate to Phe ($K_{\text{pep}}^\text{max} = 28$ s$^{-1}$), Ile ($K_{\text{pep}}^\text{max} = 22$ s$^{-1}$), and Ala ($K_{\text{pep}}^\text{max} = 25$ s$^{-1}$), smallest for the slow aa-tRNAs cognate to Asn ($K_{\text{pep}}^\text{max} = 11$ s$^{-1}$) and Gly ($K_{\text{pep}}^\text{max} = 13$ s$^{-1}$), while the classification of Pro-tRNA$^{\text{Pro}}$ as fast or slow was uncertain due to the larger error in the estimate of $K_{\text{pep}}^\text{max}$ in this case (Table 1, column 4).

MD Simulated $pK_a$-Values of A-Site Bound Aminoacyl-tRNAs. In order to examine the relation between the $pK_a^\text{obs}$-values (Table 1, column 3) and the physical process of ionizing the a-amino group in the ribosomal A site, we carried out MD simulations of the
CCA end of the six different aa-tRNAs in both the neutral and protonated form in bulk water as well as on the ribosome with a dipeptide bound to the P-site tRNA (7). We used the LIE method to estimate the pKₐ-shifts, $\Delta pK_a = pK_{a,ribo} - pK_{a,el}$, of the ribosome-bound aa-tRNAs relative to their bulk solution values in terms of electrostatic and nonpolar interaction energy contributions to the neutral and ionized forms of their $\alpha$-amino groups. In this model, the slope $\Delta \Delta pK_{a,calc}$ of the straight line that defines $\Delta pK_a$ from these standard energies is given by (see SI Text):

$$-(RT\log 10)\Delta pK_{a,calc} = \alpha \Delta \left(\Delta U_{vdw}\right) + \beta \Delta \left(\Delta U_{el}\right).$$  \[2\]

Here, $\Delta \left(\Delta U_{el}\right) = \Delta \left(\Delta U_{el}\right)_{ribo} - \Delta \left(\Delta U_{el}\right)_{aq}$ and $\Delta \left(\Delta U_{vdw}\right) = \Delta \left(\Delta U_{vdw}\right)_{ribo} - \Delta \left(\Delta U_{vdw}\right)_{aq}$, where $\Delta \left(\Delta U_{el}\right)_{ribo}$ and $\Delta \left(\Delta U_{el}\right)_{aq}$ are the differences in electrostatic energy between the ionized and neutral forms of ribosome-bound and free aa-tRNA, respectively, while $\Delta \left(\Delta U_{vdw}\right)_{ribo}$ and $\Delta \left(\Delta U_{vdw}\right)_{aq}$ are the corresponding differences for the van der Waals energy. The parameter $\beta$ is a scaling constant that relates electrostatic potential energy differences to the corresponding free energies and it includes the standard linear response factor of 1/2 as well as all contributions to the effective dielectric constant that are not represented by the microscopic simulation model of the PTC. $\alpha = 0.18$ is a standard scaling factor relating van der Waals potential energy differences, $\Delta \left(\Delta U_{vdw}\right)$, to nonpolar free energy contributions [see, e.g., (29)] and $RT\log 10 = 1.34$ at 20°C.

The $pK_a^{obs}$-values, operationally defined from the pH-dependence of the rate, $k_{pep}$, of peptidyl transfer on the ribosome as described in the previous section, are all downshifted in relation to the experimentally estimated $pK_a$-values, $pK_a^{el}$, of the $\alpha$-amino groups of aa-tRNAs in bulk solution (Table 1). If the chemistry of peptidyl transfer, rather than a preceding step, is rate limiting for $k_{pep}$ (Fig. 1), one would expect a strong correlation between $pK_a$-shifts calculated according to Eq. 2 above and the experimentally determined $\Delta pK_{a}^{obs} = pK_{a}^{obs} - pK_{a}^{el}$ values (Table 1). If, in contrast, a step preceding the chemistry of peptidyl transfer is rate limiting for $k_{pep}$, no such correlation would be expected a priori.

We plotted the empirical $\Delta pK_{a}^{obs}$-values for the six different aa-tRNAs vs. the MD-estimated $\Delta pK_{a,calc}$-values for the six tRNAs...
with the unknown dielectric scaling constant, \( \beta \), as a fitted parameter (Fig. 4). The figure reveals a remarkably strong, positive correlation between the \( \Delta pK_{a}^{\text{calc}} \) and \( \Delta pK_{a}^{\text{obs}} \)-values.

**Discussion**

We have studied the pH-dependence at 20 °C of the rate constant, \( k_{\text{pep}} \), for fMet transfer from initiator tRNA in the P site to each of one of six aa-tRNAs cognate to Asn, Phe, Ile, Ala, Gly, or Pro in the A site of the ribosome. \( k_{\text{pep}} \) defined as the inverse of the average time, \( \tau_{\text{pep}} \), for aa-tRNA to receive fMet after hydrolysis of GTP on EF-Tu (Fig. 1), displays distinct pH-dependence for all six tRNAs (Fig. 3 B–G). The pH-dependence of the peptidyl transfer reaction, quantified as the pH-value, \( pK_{a}^{\text{obs}} \), at which \( k_{\text{pep}} \) is half its maximal value at high pH, \( pK_{a}^{\text{max}} \), varies among the six aa-tRNAs by almost two pH units (Table 1, column 3). There is, at the same time, a positive correlation between the \( pK_{a} \)-value of the \( \alpha \)-amino group of an aa-tRNA in bulk water (Table 1, column 2), \( pK_{a}^{\text{obs}} \), and its \( pK_{a}^{\text{calc}} \)-value, suggesting that the pH-dependence of \( k_{\text{pep}} \) reflects inhibition of peptide bond formation due to protonation of the \( \alpha \)-amino group of the A-site aa-tRNA. Furthermore, plots of \( \log_{10}(k_{\text{pep}}) \) vs. pH in the Pro and Gly cases give straight lines with slopes very close to one in the low pH range (Fig. 3H), suggesting titration of a single proton for peptidyl transfer to Gly-tRNA\( ^{\text{Gly}} \) and Pro-tRNA\( ^{\text{Pro}} \) (11). From this result, we propose that titration of the \( \alpha \)-amino groups of Gly and Pro is the sole explanation for the pH-dependence of the rate of peptidyl transfer to these aa-tRNAs (Fig. 2). Because also the pH-dependence of \( k_{\text{pep}} \) of the other four tRNAs is accounted for by single proton titrations of their \( \alpha \)-amino groups, we suggest that the pH-dependence of \( k_{\text{pep}} \) has the same origin for all six tRNAs. It is, we suggest, not likely that this variegated spectrum of \( pK_{a}^{\text{obs}} \)-values and its correlation with the spectrum of \( pK_{a}^{\text{calc}} \)-values associated with its aa-tRNAs is caused by proton titration on any other functional group in the ribosome or by different tRNA-bodies. Yet another hypothesis, by which the rate of accommodation of aa-tRNA in the A site is severely inhibited by protonation of its amino group, so far lacks a clear mechanistic interpretation.

A distinct pH-dependence of the rate of peptidyl transfer to native aa-tRNAs in the ribosomal A site has not been observed before (15). The absence of pH-dependence has been rationalized as due to rate limiting accommodation of aa-tRNA in the A site, masking the (expected) pH-dependence of the rate constant, \( k_{\text{pt}} \) (see Fig. 1), for the chemistry of peptide bond formation (15). In fact, the pH-dependence of the peptidyl transfer reaction has previously been observed only for small aa-tRNA-analogs, like puromycin, C-puromycin, CC-puromycin (23, 30, 31), or puromycin analogs (32). For these, the pH-dependence is complex, with the effect of two protons on the rate of peptidyl transfer to puromycin at 37 °C (23, 30) as well as at 20 °C (31). The effect of two protons or one proton was observed for C-puromycin at 37 °C (30) or 20 °C (31), respectively. Furthermore, the rate of peptidyl transfer to CC-puromycin displayed a qualitatively different and much weaker pH-dependence (30).

In contrast, the present findings show that the rate constant for the chemistry of peptidyl transfer, \( k_{\text{pt}} \), in Fig. 1, can be directly assessed in kinetic experiments with native aa-tRNAs performed at pH-values below the \( pK_{a}^{\text{obs}} \)-values reported in Table 1: in these experimentally accessible pH-regimes the chemistry of peptidyl transfer is slower than the accommodation of aa-tRNA in the A site. An interesting question is now whether the chemistry of peptidyl transfer is slower than preceding steps on the pathway from GTP hydrolysis only at low pH-values. Or, alternatively, if also the maximal rate constant for peptide bond formation (\( k_{\text{pt}}^{\max} \)) in the high pH range is rate limited by the chemistry of peptidyl transfer for some or all aa-tRNAs. The answer to this question depends on the cause of the varying downshifts of the kinetically determined \( pK_{a}^{\text{obs}} \)-values in relation to the \( pK_{a}^{\text{calc}} \)-values for aa-tRNAs in bulk water (Compare columns 2 and 3 in Table 1).

In our kinetic analysis based on average times, \( pK_{a}^{\text{obs}} \) is determined by the \( pK_{a}^{\text{calc}} \)-value, \( pK_{a}^{\text{rib}} \), of the \( \alpha \)-amino group of a ribosome-bound aa-tRNA through (SI Text):

\[
pK_{a}^{\text{obs}} = pK_{a}^{\text{calc}} + \log_{10}(k_{\text{pt}}^{\max}/k_{\text{pt}}^{\text{calc}}).
\]

[3]

Here, \( k_{\text{pt}}^{\max} \) is the high pH-limit of the rate constant, \( k_{\text{pt}} \), for the chemistry of peptidyl transfer, i.e., when the \( \alpha \)-amino group is neutral (Fig. 2) and \( pK_{a}^{\text{calc}} \) is the corresponding high pH-limit of \( pK_{a}^{\text{calc}} \)-values, a strong correlation of the \( \alpha \)-amino groups of the aa-tRNAs in bulk water (Table 1) are given by:

\[
\Delta pK_{a}^{\text{calc}} = pK_{a}^{\text{calc}} - pK_{a}^{\text{calc}} = \Delta pK_{a} + \log_{10}(k_{\text{pt}}^{\max}/k_{\text{pt}}^{\text{calc}}).
\]

[4]

This expression shows that if a putative downshift, \( \Delta pK_{a} \), of the \( pK_{a} \)-value of the amino group of an aa-tRNA as it moves from bulk water to the A site of the ribosome, were known, the ratio \( k_{\text{pt}}^{\text{calc}}/k_{\text{pt}}^{\max} \) could be determined from \( \Delta pK_{a}^{\text{calc}} \). This ratio could then be used to decide the extent to which the overall peptidyl transfer reaction is rate limited by aa-tRNA accommodation and other steps preceding the chemistry of peptide bond formation. Because \( \Delta pK_{a} \) cannot be directly measured, we have used MD techniques and the LIE approximation (29) to estimate \( \Delta pK_{a} \) according to Eq. 2. When the estimated values of \( \Delta pK_{a} \), \( \Delta pK_{a}^{\text{calc}} \), are plotted vs. the \( \Delta pK_{a}^{\text{obs}} \)-values, a strong correlation is seen (Fig. 4). The major part of the correlation stems from the electrostatic energy contribution, \( \Delta\Sigma(U_{el}) \) (Fig. S2), while the nonpolar contribution \( \Delta\Sigma(U_{vdw}) \) mainly affects the \( pK_{a} \)-shift of Phe-tRNA\( ^{\text{Phc}} \). The structural explanation for the different observed \( pK_{a} \)-shifts is straightforward, as judged from the average MD structures (Fig. 5). That is, water molecules are generally occluded from the volume surrounding the protonated amino group in the PTC, due to the position of the amino acid side chain.
in the A site. This water occlusion leads to $k_\text{dip}$ downshifts because desolvation destabilizes the NH$_2$ in relation to the NH$_3$ state (Fig. 5A). The only exception is Gly-tRNA$^{\text{Gly}}$, for which a water molecule can enter the volume normally occupied by the C$_\beta$ atom, thus providing more solvation of the amine and thus explaining why Gly had the smallest $k_\text{dip}$-shift (Fig. 5B). In the case of Pro-tRNA$^{\text{Pro}}$, on the other hand, because of the cyclic structure of Pro the volume is already more blocked for the side chain in bulk water than for most side chains, so that the desolvation effect in the PTC is not as pronounced and this leads to a smaller $k_\text{dip}$-shift than for most side chains. Also, Asn-tRNA$^{\text{Asn}}$ is somewhat special because the side chain amide carbonyl group can form a favorable interaction (H-bond) with its protonated N terminus, thus somewhat attenuating the desolvation effect. The remaining three aa-tRNAs (Phe, Ala, and Ile) have the largest downshifts because of the lack of compensating interactions with the amino group.

Although the absolute value calibration of the MD calculations depended on a fitting of the unknown dielectric scaling constant, $\beta$, in Eq. 2 above, a straightforward interpretation of the observed downshifts, $\Delta pK_{\text{obs}}$, is that they mainly reflect amino acid specific downshifts, $\Delta pK_\alpha$, in the $k_\text{dip}$-values of aa-tRNAs as they become ribosome bound. This interpretation is in line with the fact that the already unnormalized, $\beta$-dependent electrostatic potential energy differences, $\Delta U(U_\beta)$, correlate significantly with the observed downshifts (Fig. S2). The correspondence of $\Delta pK_{\text{obs}}$ to $\Delta pK_\alpha$ would imply that $k_{\text{dip}}^{\text{max}} = k_{\text{dip}}^{\text{max}}$, meaning that the chemistry of peptide transfer would dominate the overall rate of peptide transfer for all six aa-tRNAs also at high pH-values.

However, this conclusion is controversial, because it is generally assumed that accommodation of native aa-tRNAs in the A site is rate limiting for peptide transfer (18–21). Evidence for rate limiting A-site accommodation of aa-tRNA was originally based on stopped-flow experiments, showing that rapid mixing of EF-Tu·GTP in complex with fluorescence-labeled Phe-tRNA$^{\text{Phe}}$ with ribosomes containing fMet-tRNA$^{\text{Met}}$ or deacylated tRNA$^{\text{Met}}$ in the P site leads to the same fast fluorescence change, interpreted as GTPase activation of EF-Tu, followed by the same slow fluorescence change, interpreted as Phe-tRNA$^{\text{Phe}}$ accommodation in the A site. From the equivalence of the rate of the slow fluorescence change and the overall rate of peptide bond formation ($k_{\text{dip}}$ in Fig. 1), accommodation was concluded to be rate limiting for $k_{\text{dip}}$ (16, 17). In those (16, 17) and similar (15) experiments the $k_{\text{dip}}$-values were much smaller than $k_{\text{dip}}$-values reported by us (22), suggesting that a rate limiting accommodation step had been removed in our optimized buffer system, thereby allowing for direct kinetic studies of the chemistry of peptide bond formation. Very recent quench-flow experiments (33) now confirm the previously reported (22) large maximal values of $k_{\text{dip}}$ at saturating ternary complex concentration. The authors (33) also performed stopped-flow experiments using fluorescence-labeled Phe-tRNA$^{\text{Phe}}$ at a single ribosome concentration in excess over ternary complex and found a $k_{\text{dip}}$ value identical to a fluorescence relaxation rate about five times smaller than the maximal value of $k_{\text{dip}}$ at saturating concentration of ternary complexes. The slow fluorescence relaxation was ascribed to accommodation and the authors argued that its similarity with $k_{\text{dip}}$ proves accommodation limited peptide transfer (33). However, the accommodation rate constant must always be larger than the maximal value of $k_{\text{dip}}$. Therefore, the observations in (33) do not support the assignment of the slow fluorescence change to the aa-tRNA accommodation step. Further experiments will be needed to define the exact contributions of aa-tRNA accommodation and the chemistry of peptide bond formation to the overall kinetics of peptide transfer.

Conclusions

We have found that ribosomal peptide transfer to Gly-tRNA$^{\text{Gly}}$ and Pro-tRNA$^{\text{Pro}}$ at pH 7.5 is rate limited by the chemistry of peptide transfer, rather than by a preceding step following GTP hydrolysis on EF-Tu. A straightforward interpretation of the results of our MD simulations, revealing amino acid specific downshifts of the $pK_\alpha$-values of aa-tRNAs upon A-site binding, is that the chemistry of peptide bond formation is rate limiting in a pH-independent manner for all six of the studied aa-tRNAs. Resolution of the apparent contradiction between this strong conclusion from the present work and the fluorescence based conclusion that peptide transfer to Phe-tRNA$^{\text{Phe}}$ is accommodation limited at physiological pH will require more detailed kinetic experiments. In any case, our results demonstrate that careful choices of pH in the 6–8 unit range open kinetic windows for direct study of the chemistry of peptide bond formation for six aa-tRNAs in an approach that is extendable to a much larger set of aa-tRNAs. Such kinetic windows are essential for characterization of the phenotypic effects of mutations in ribosomal RNA and protein with implications for cell physiology and molecular evolution.

Materials and Methods

Reagents. 70S ribosomes (E. coli strain MRE 600), synthetic mRNAs, initiation factors, elongation factors, and radiolabeled fMet-tRNA$^{\text{Met}}$ were prepared according to ref. 22 and references therein. tRNAs were from Sigma-Aldrich and Chemical Block (Russia). Radioactive compounds were from GE Healthcare and all other chemicals were from Merck or Sigma-Aldrich. All experiments were carried out in polymyx buffer [95 mM KCl, 5 mM NH$_4$Cl, 5 mM Mg(OAc)$_2$, 0.5 mM CaCl$_2$, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate and 1 mM dithioerythritol (DTE)] (34).

Initiated 70S Ribosomes and Ternary Complexes. Initiated 70S ribosomes, carrying fMet-tRNA$^{\text{Met}}$ (fMet or $^5$HfMet) in P site and displaying either of the codons U(UU) (Phe), CCC (Pro), AAC (Asn), GGC (Gly), GCA (Ala), or AUG (Ile) in A site, were prepared by incubating 70S ribosomes (80–85% active in dipeptide formation), fMet-tRNA$^{\text{Met}}$ (1.5 times the ribosome concentration), mRNA, IF1, IF3 (all in twice the ribosome concentration), and IF2 (same concentration as ribosomes) for 10 min at 37 °C. Ternary complexes were prepared by first equilibrating EF-Tu alone with $^5$H(GDP) (1:1 with EF-Tu) for 15 min at 37 °C, and then incubating it for 20 min at 37 °C in a mixture consisting of the amino acid of interest (400 μM), the corresponding aa-tRNA synthetase (2 units/μL) and tRNA (Purified tRNA$^{\text{Phe}}$ for fMetPhe dipeptide formation, bulk tRNA in all other cases). In addition, the ribosome mix contained ATP (1 mM) and GTP (1 mM), and the ternary complex mix contained ATP (2 mM) but no extra GTP. Both mixtures contained phosphoenolpyruvate (PEP) (10 mM), pyruvate kinase (PK) (50 μg/mL), and myokinase (MK) (2 μg/mL). For the fMetPhe dipeptide experiments, where purified tRNA$^{\text{Phe}}$ was available, the concentration of ribosomes (1 μM final) was less than the concentration of ternary complex (4 μM final), whereas in all other experiments, the concentration of ribosomes (1–2 μM final) was higher than that of ternary complex (0.5–1 μM final).

pH Adjustments. The two reaction mixtures were prepared in pH adjusted buffer, before adding the kinetic ribosomes. pH of the ribosome mixture was checked (using a Hamilton Mininutrode) and corrected if necessary by the addition of KOH (0.5 M) or HCl (1 M). After adding the ribosomes, pH of the two mixtures was equalized if necessary by the addition of KOH (0.5 M) or HCl (1 M) to the ternary complex mix.

Fig. 5. Snapshots from MD simulations with Gly-Phe-tRNA$^{\text{Phe}}$ in P site and Phe-tRNA$^{\text{Phe}}$ (A) or Gly–tRNA$^{\text{Gly}}$ (B) in A site. The red spheres represent water molecules.

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Rapid Quench-Flow Experiments. The two mixtures were rapidly mixed in equal volumes in a temperature controlled quench-flow instrument (RFQ-3, KinTek Corp.) as described previously (22). The quenched samples were centrifuged for 15 min at 20,800 x g and the [3H]GTP and [3H]GDP in the supernatants were separated and analyzed using either TLC (35) or a MonoQ ion-exchange column (GE Healthcare) with an on-line scint counter. Supernatants were separated and analyzed using either TLC (35) or a MonoQ ion-exchange column (GE Healthcare) with an on-line scint counter (IRAM3; INUS Inc.) (24). The extent of dipeptide formation was analyzed by RP-HPLC according to ref. 36.

**pKₐ in Bulk Water.** The pKₐ-values of the α-amino group of different aa-tRNAs in solution at 20 °C were extrapolated from measured values of the pKₐ of free amino acids and amino acid esters. It has previously been shown that the α-amino pKₐ is very similar for tRNA-bound amino acids and more simple amino acid esters (14). From the temperature dependence of the pKₐ for several amino acid methyl and ethyl esters (12, 13), the pKₐ at 20 °C was calculated to be 0.10 and 0.15 units higher than at 25 °C. The pKₐ of the amino acid methyl and ethyl esters are downshifted between 2.0 and 2.2 units in relation to the corresponding amino acids (12, 13). As neither Asn nor Pro were included in the studies, we have for all six aa-tRNAs estimated the α-amino pKₐ at 20 °C (Table 1) as a downshift of 0.2 units from the pKₐ of free amino acids at 25 °C (11).


**MD Simulations.** The calculations were based on the crystallographic coordinates of the 505 Haloarcula marismortui ribosome with the bound peptidyl transfer transition state analog RAP, PDB code 1VOQ (4). The RAP moiety was converted to an S-enantiomeric tetrahedral intermediate corresponding to CCA-Phe in the A site and CCA-Phe-Gly in the P site where the terminal amino group was kept neutral. The system was prepared using essentially the same protocols as described previously (7, 8) and consisted of all residues with atoms within 20 Å of the P-site carboxyl carbon and was solvated using the program Q (37). All Sr²⁺ ions were converted to Mg²⁺ ions and phosphate linkages and ions outside 15 Å were neutralized as described earlier. The system was relaxed to the reactant state with the aa-tRNA with a free amine in the A site and dipeptide-tRNA ester in the P site. Five additional systems were constructed in this way with A-site aa-tRNAs corresponding to the amino acids Ala, Asn, Gly, Ile, and Pro. MD simulations were performed (SI Text) using the program Q (37) at 300 K with the CHARMM22 force field (38).

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Supporting Information

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SI Text

SI Methods. Data evaluation. When a ternary complex, consisting of EF-Tu, aa-tRNA, and GTP, reacts with an initiated 70S ribosome, a series of events take place (1). As shown in Fig. 3.4 the time evolution of peptidyl bond formation in the single-round experiments used here, has a sigmoidal look, due to two or more chemical steps with kinetics in the same time scale. As we have shown earlier, it is correct to evaluate the results from this type of experiment using mean-time calculations (2). By simply integrating the area, up to one, above a normalized time course of e.g., dipeptide formation, the mean-time of the overall reaction is achieved, i.e.:

$$\tau_{\text{dip}} = \int_0^\infty \left( 1 - \frac{\text{dip}(t)}{\text{dip}(\infty)} \right) \, dt.$$ \[S1\]

Similarly, the average time for GTP hydrolysis, $\tau_{\text{GTP}}$, is:

$$\tau_{\text{GTP}} = \int_0^\infty \left( 1 - \frac{\text{GDP}(t)}{\text{GDP}(\infty)} \right) \, dt.$$ \[S2\]

For the simplified scheme in Fig. 1, the difference between $\tau_{\text{dip}}$ and $\tau_{\text{GTP}}$, $\tau_{\text{pep}}$, can be expressed from the individual rate constants as:

$$\tau_{\text{pep}} = \tau_{\text{dip}} - \tau_{\text{GTP}} = \frac{1}{k_\text{uu}} + \frac{1}{k_\text{ac}} + \frac{1}{k_\text{pt}}.$$ \[S3\]

If release of EF-Tu-GDP occurs in parallel with accommodation (1), then the term $1/k_\text{uu}$ is excluded from the right hand side of Eq. S3. In our experiments we were interested in the steps subsequent to hydrolysis of EF-Tu-bound GTP. Accordingly we monitored the time evolution of GTP hydrolysis and dipeptide formation in the very same experiment (Fig. 3.4). The time course of GTP hydrolysis, including binding of T3 to 70S ribosomes, codon-anticodon recognition, GTPase activation, and GTP hydrolysis (1), could in all cases be excellently fitted to a single exponential expression. With bg a constant background, the mean-time for GTP hydrolysis, $\tau_{\text{GTP}}$, was estimated from:

$$\text{GTP}(t) = P \left( 1 - e^{-\tau_{\text{GTP}}} \right) + \text{bg}.$$ \[S4\]

The standard deviation, $\sigma_{\tau_{\text{GTP}}}$, of $\tau_{\text{GTP}}$ was provided by the fitting program [Levenberg-Marquardt algorithm fit using Origin (OriginLab Corp.)]. For dipeptide formation, the curves were fitted to a two-step reaction model containing the mean-time for dipeptide formation, $\tau_{\text{dip}}$, as one of the parameters:

$$\text{dip}(t) = \frac{R_0}{k_1 - \frac{1}{\tau_{\text{dip}} - 1/k_1}} \left( 1 - e^{-\frac{t}{\tau_{\text{dip}}}} \right) + \frac{1}{\tau_{\text{dip}} - 1/k_1} \left( 1 - e^{-1/k_1 \cdot t} \right) + \text{bg}.$$ \[S5\]

The use of $\tau_{\text{dip}}$ rather than the second rate constant in the model allowed us to obtain an estimate of the standard deviation, $\sigma_{\tau_{\text{dip}}}$, of $\tau_{\text{dip}}$ directly from the fit as in the case of $\sigma_{\tau_{\text{GTP}}}$. The mean-time for all the steps subsequent to GTP hydrolysis up to peptidyl transfer, $\tau_{\text{pep}}$, was estimated as:

$$\tau_{\text{pep}} = \tau_{\text{dip}} - \tau_{\text{GTP}}.$$ \[S6\]

The standard deviation, $\sigma_{\tau_{\text{pep}}}$, of $\tau_{\text{pep}}$ was estimated as:

$$\sigma_{\tau_{\text{pep}}} = \sqrt{\sigma_{\tau_{\text{dip}}}^2 + \sigma_{\tau_{\text{GTP}}}^2}.$$ \[S7\]

The corresponding rate reaction, $k_{\text{pep}}$, and its standard deviation, $\sigma_{k_{\text{pep}}}$, were estimated as:

$$k_{\text{pep}} = \frac{1}{\tau_{\text{pep}}}, \quad \sigma_{k_{\text{pep}}} = \frac{\sigma_{\tau_{\text{pep}}}}{\tau_{\text{pep}}}.$$ \[S8\]

All experiments were repeated at least twice. Maximum likelihood estimates of average $k_{\text{pep}}$ values were obtained from single experiment variances ($w_i = 1/\sigma^2_{k_{\text{pep}}}$) by minimizing the $\chi^2$ function:

$$\chi^2 = \sum_{i=1}^n w_i \cdot (k_{\text{pep}(i)} - k_{\text{pep}})^2.$$ \[S9\]

with respect to $k_{\text{pep}}$. From this expression we obtained:

$$k_{\text{pep}} = \frac{\sum_{i=1}^n w_i \cdot k_{\text{pep}(i)}}{\sum_{i=1}^n w_i}, \quad \sigma_{k_{\text{pep}}} = \frac{1}{\sqrt{\sum_{i=1}^n w_i}}.$$ \[S10\]

According to the present results with peptidyl transfer between native tRNAs, only one reacting group is being protonated when pH is changed near physiological pH, i.e., the $\alpha$-ammonium proton on the aminoacyl-tRNA. The pH dependence of peptidyl transfer, $k_{\text{pt}}$, in Fig. 1, is therefore (3):

$$k_{\text{pt}} = \frac{k_{\text{max}}}{1 + 10^{(pK_{\text{a}} - \text{pH})/pK_{\text{a}}}}.$$ \[S11\]

Here, the rate at complete protonation is assumed to be zero, in line with the current understanding of aminolysis (4, 5). From our experiments, we calculate the compounded rate constant, $k_{\text{pep}}$, of all steps subsequent to GTP hydrolysis as the inverse of the time $\tau_{\text{pep}}$ (See Fig. 3.4 and Eq. S8 above). The parameters $k_{\text{max}}$ and $pK_{\text{a}}$, in Table 1, were estimated by fitting the experimentally observed pH variation of $k_{\text{pep}}$ to the function:

$$k_{\text{pep}} = \frac{k_{\text{max}}}{1 + 10^{(pK_{\text{a}} - \text{pH})/pK_{\text{a}}}}.$$ \[S12\]

The observed $pK_a$ value is related to the $pK_a$ value of the $\alpha$-amino group of a ribosome bound aa-tRNA, $pK_{\text{a}}$, and the “downshift term” log$_10$($k_{\text{max}}$/$k_{\text{pep}}$) through:

$$pK_{\text{a}} = pK_{\text{a}} + \log_{10}\left( \frac{k_{\text{max}}}{k_{\text{pep}}} \right).$$ \[S13\]

The resolution of our kinetic experiments allows precise estimates of the average times $\tau_{\text{dip}}$, $\tau_{\text{GTP}}$, and $\tau_{\text{pep}}$, but not the downshift ratio $k_{\text{max}}/k_{\text{pep}}$ in Eq. S13 (see Discussion in the main text). When $pK_{\text{a}}$ is equal to the $pK_{\text{a}}$-value of aminoacyl-tRNAs or their analogues in bulk water, $pK_{\text{a}}$, $k_{\text{max}}$ can be calculated from observed data and Eq. S13 as:

$$k_{\text{max}} = k_{\text{pep}} \cdot 10^{pK_{\text{a}} - pK_{\text{a}}}.$$ \[S14\]
The compounded rate constant of the steps between GTP hydrolysis and the chemistry of peptide bond formation can be calculated as:

\[
\frac{1}{1/k_{\text{pop}}^{\text{max}}} - \frac{1}{1/k_{\text{pt}}^{	ext{max}}}. \tag{S15}
\]

**MD-simulated pKₐ-values of A-site bound aminoacyl-tRNAs.** In order to examine the relation between the pKₐ-values (Table 1, column 3) and the physical process of ionizing the α-amino group in the ribosomal A site we carried out molecular dynamics (MD) simulations of the CCA end of the six different aa-tRNAs in both the neutral and protonated form in bulk water as well as in the ribosome with a dipeptide bound to the P-site tRNA. The systems including solvent and ions were prepared using essentially the same protocols as described previously (6, 7) and comprised all residues with atoms within 20 Å of the P-site carbonyl carbon. Atoms within the system boundary were fully mobile while atoms outside the 20 Å radius were restrained to their initial positions with a 100 kcal/(mol·Å²) harmonic force constant. No nonbonded interactions outside or across the boundary was calculated and water molecules close to the boundary were restrained to reproduce the correct density and polarization (8). All nonbonded interactions were calculated for the α-amino while for other interactions a multipole expansion treatment (9) of long-range electrostatics (beyond 10 Å) was employed. The different systems were heated from 1 K to 300 K in a step wise manner with initial random velocities taken from a Maxwell-Boltzmann distribution and the temperature was kept constant by coupling to an external heat bath. The MD simulations used a time step of 1 fs and the systems were equilibrated at 300 K for at least 300 ps before data collection. Data were collected every 0.1 ps for all systems during subsequent MD simulations of 4 ns for other interactions a multipole expansion treatment (9) of long-range electrostatics (beyond 10 Å) was employed. The different systems were heated from 1 K to 300 K in a step wise manner with initial random velocities taken from a Maxwell-Boltzmann distribution and the temperature was kept constant by coupling to an external heat bath. The MD simulations used a time step of 1 fs and the systems were equilibrated at 300 K for at least 300 ps before data collection. Data were collected every 0.1 ps for all systems during subsequent MD simulations of 4 ns each. The same set of simulations were performed for each of the six aa-tRNAs with the α-amino protonated. Finally, an identical simulation scheme was repeated for each of the six amino acid esters in a 20 Å sphere of water, with the α-amino both neutral and protonated.

The pKₐ-shifts of the ribosomal bound aa-tRNAs, relative to their unperturbed values in aqueous solution, are given by the thermodynamic cycle corresponding to moving the neutral and protonated aa-tRNA species from water to the ribosomal A-site (Scheme S1). Here, ΔGₑₑ and ΔGₚₚ are the differences in standard free energy between the protonated and deprotonated forms of the α-amino groups of aminoacyl-tRNAs in bulk water (aq) and ribosomal A site (ribo), respectively. It follows directly from the definitions of pKₐ-values for ribosome bound, pKₐₚ, and free, pKₐₑₑ, aminoacyl-tRNAs that:

\[
\text{RT} \log 10(pK_{a}^{\text{ribo}} - pK_{a}^{\text{aq}}) = \text{RT} \log 10\Delta pK_{a}^{\text{calc}} = -(\Delta G^{\text{ribo}} - \Delta G^{\text{aq}}) = -\Delta G. \tag{S16}
\]

The absolute standard free energy differences ΔGₚₚ and ΔGₑₑ are difficult to calculate because they in principle depend on the dielectric properties of the entire solvated ribosome. However, the relative free energies between different aa-tRNAs are easier to obtain as long-range electrostatic effects can then be expected to cancel. Here, we estimate these free energy differences by the semiempirical linear interaction (LIE) method (10) which relates the average polar and nonpolar interactions between the ionizing amino acid ester and its surroundings (i.e., the potential energy differences Δ(Uₚₚ), Δ(Uₑₑ) and Δ(Uₚₚ) to the corresponding free energy contributions. This method gives a linear relation between ΔpKₐ and the average energies are obtained, where the slope, ΔpKₐ, is given by:

\[
-1.34\Delta pK_{a}^{\text{calc}} = 0.18\Delta(U_{\text{vdw}}) + \beta\Delta(U_{\text{cl}}). \tag{S17}
\]

Here, ΔUₚₚ = Δ(Uₚₚ) = Δ(Uₚₚ) + Δ(Uₚₚ) and Δ(Uₑₑ) = Δ(Uₑₑ) + Δ(Uₑₑ). β is an electrostatic scaling constant for the surroundings of the peptidyl transferase center (PTC) and 0.18 is a standard scaling factor relating van der Waals interactions (Δ(Uₑₑ)) to nonpolar free energy contributions (11), while 1.34 is RTlog(10) (at 20 °C). MD simulations were used to calculate Δ(Uₑₑ) and Δ(Uₚₚ) for all six aminoacyl-tRNAs.

Fig. S1. The normalized amount of GDP and dipeptide formed as a function of time when EF-Tu·aa-tRNA$^{35S}$/GTP ternary complexes (aa-tRNAs as indicated in the figure) react at $\sim$pH 7.5 and 20 °C with f$^{35S}$Met-tRNA$^{Met}$ initiated 70S ribosomes displaying GCA (Ala), AAC (Asn), GGC (Gly), AUC (Ile), or CCC (Pro) codon in A-site. The shaded areas represent the mean-time, $\tau_{pep}$, for all reaction steps subsequent to GTP hydrolysis up to and including peptidyl transfer.
Fig. S2. MD-simulated electrostatic potential energy differences, $\Delta \Delta U_{el}$, for the neutral and ionized form of the $\alpha$-amino group of an A-site bound or free aa-tRNA, plotted vs. experimentally observed $pK_a$ shifts, $\Delta pK_{a}^{\text{obs}} = pK_{a}^{\text{obs}} - pK_{a}^{\text{aq}}$ (Table 1). Phe-tRNA$^{\text{Phe}}$ is set as standard (0 kcal/mol) for the relative values of $\Delta \Delta (U_{el})$.

Scheme S1.

\[
\begin{align*}
\text{CCA-O-CONH}_2 & \rightarrow \text{CCA-O-CONH}_2^+ \quad \text{(water)} \\
\text{CCA-O-CONH}_2 & \rightarrow \text{CCA-O-CONH}_2^+ \quad \text{(A site)}
\end{align*}
\]