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Lack of discrimination against non-proteinogenic amino acid norvaline by elongation factor Tu from *Escherichia coli*

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Abstract

The GTP-bound form of elongation factor Tu (EF-Tu) brings aminoacylated tRNAs (aa-tRNA) to the A-site of the ribosome. EF-Tu binds all cognate elongator aa-tRNAs with highly similar affinities, and its weaker or tighter binding of misacylated tRNAs may discourage their participation in translation. Norvaline (Nva) is a non-proteinogenic amino acid that is activated and transferred to $tRNA^{Leu}$ by leucyl-tRNA synthetase (LeuRS). No notable accumulation of Nva-$tRNA^{Leu}$ has been observed *in vitro*, because of the efficient post-transfer hydrolytic editing activity of LeuRS. However, incorporation of norvaline into proteins in place of leucine does occur under certain conditions *in vivo*. Here we show that EF-Tu binds Nva-$tRNA^{Leu}$ and Leu-$tRNA^{Leu}$ with similar affinities, and that Nva-$tRNA^{Leu}$ and Leu-$tRNA^{Leu}$ dissociate from EF-Tu at comparable rates. The inability of EF-Tu to discriminate against norvaline may have driven evolution of highly efficient LeuRS editing as the main quality control mechanism against misincorporation of norvaline into proteins.

Keywords

EF-Tu; norvaline; non-proteinogenic amino acids; aminoacyl-tRNA synthetases; leucyl-tRNA synthetase; mistranslation

INTRODUCTION

Bacterial elongation factor Tu (EF-Tu) delivers elongator aminoacyl-tRNAs (aa-tRNA) to the ribosome, where they are utilized in protein synthesis.[1,2] EF-Tu belongs to a group of G-binding proteins that alternate between inactive and active forms by a mechanism involving the exchange of GDP with GTP. The GTP-bound form (EF-Tu:GTP) binds all elongator aa-tRNAs with very similar affinities in the nanomolar range,[3–5] thus enabling a consistent rate of protein translation. In contrast, the GDP-bound form (EF-Tu:GDP) possesses rigorously decreased affinity for aa-tRNAs.[6–8] Coupling of aa-tRNA binding by EF-Tu with its GTP/GDP cycle is important for accurate recognition of aa-tRNAs on the ribosome. Binding of the ternary EF-Tu:GTP:aa-tRNA complex to a cognate mRNA codon triggers the GTP-ase activity, which releases cognate aminoacylated tRNA for binding in the ribosomal A site.[2]

Interestingly, EF-Tu:GTP displays substantial specificity for both the amino acid and the tRNA portions of its aa-tRNA ligands.[4,5,9] The nearly uniform binding affinity observed for tRNAs aminoacylated with their cognate amino acid arises from thermodynamic compensation in binding the tRNA body and the esterified amino acid[3–5]. Thus, weak-

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binding amino acids are esterified to cognate tRNAs that bind EF-Tu tightly, while tight-binding amino acids are matched with tRNAs that bind EF-Tu weakly. Strong thermodynamic contribution of either the esterified amino acid or the tRNA body, therefore, compensates for the weak thermodynamic contribution of another portion of the cognate aa-tRNA pair. In contrast, tRNAs acylated with non-cognate amino acids (misacylated tRNAs) bind EF-Tu with a broad range of affinities, varying from 60-fold weaker to 120-fold tighter than cognate aa-tRNAs. This allows EF-Tu to discriminate against at least some misacylated tRNAs, discouraging their further participation in translation.[10–12]

Aminoacyl-tRNA synthetases (aaRSs) covalently link amino acids with cognate tRNAs in a two-step synthetic reaction that proceeds *via* an aminoacyl-adenylate intermediate.[13] Norvaline (Nva) is a non-proteinogenic amino acid that may increase in concentration to as high as 1 mmol dm^{-3} during unlimited growth of *Escherichia coli* on glucose after a down-shift in oxygen levels.[14] Interestingly, a low but readily detectable incorporation of norvaline for leucine was observed in recombinant human hemoglobin produced in *E. coli*, and the extent of misincorporation strongly correlated with the ratio of free norvaline to leucine.[15] Thus, under conditions that promote norvaline accumulation, leucyl-tRNA synthetase (LeuRS) catalyzes formation of Nva-$tRNA^{Leu}$ *in vivo*. Norvaline possesses a linear three-carbon side chain that cannot be excluded from the LeuRS amino acid binding site on steric grounds (Figure 1A), preventing efficient discrimination in the synthetic reactions alone. Therefore, to achieve the accuracy required for protein synthesis, LeuRS possesses intrinsic hydrolytic editing activities to exclude norvaline. It is now well established that many aaRSs are incapable of efficient discrimination between cognate and structurally similar non-cognate amino acids in the synthetic reactions.[16,17] These enzymes therefore evolved editing mechanisms to hydrolyze non-cognate intermediates (pre-transfer editing) and/or misacylated tRNAs (post-transfer editing).[16]

Work in our and other laboratories has shown that *E. coli* LeuRS indeed treats norvaline as a reasonably good substrate in the synthetic reactions; k_{cat}/K_m in activation is decreased only 100-fold as compared with leucine,[18,19] while the rate of aminoacyl transfer to tRNA is identical.[19] Despite this, we did not observe significant steady-state accumulation of Nva-$tRNA^{Leu}$ *in vitro* due to the rapid clearance of Nva-$tRNA^{Leu}$ by the post-transfer editing activity[19] located on the separate editing domain known as the CP1 (connective peptide 1) domain.[20,21] We also demonstrated that dissociation of Nva-$tRNA^{Leu}$ followed by rebinding and subsequent hydrolysis is a competent kinetic pathway.[19] To test if any Nva-$tRNA^{Leu}$ that evades hydrolytic correction is a substrate for ribosomal protein synthesis, its interaction with *E. coli* EF-Tu:GTP was studied at low (4 °C) and physiological (37 °C) temperatures. Using slightly modified versions of the ribonuclease (RNase) protection[3,4,22] and non-enzymatic hydrolysis protection assays,[23,24] we show that *E. coli* EF-Tu does not differentiate between Leu-$tRNA^{Leu}$ and Nva-$tRNA^{Leu}$ at either temperature. The lack of discrimination against norvaline by EF-Tu highlights the importance of rapid hydrolytic correction by LeuRS, demonstrating that it provides the main line of defense against misincorporation of norvaline into proteins. Enhanced understanding of the molecular events that maintain selectivity against non-proteinogenic and/or non-natural amino acids may advance the engineering of proteins with desired features.[25,26]

EXPERIMENTAL

Cloning, overexpression and purification of N-His_6-EF-Tu

The *E. coli tufB* gene was PCR-amplified as a *Bam*HI-*Xho*I cassette and cloned into expression vector pPROEXHtb. This construct enables overexpression of EF-Tu with an N-terminal (His_6)-TEV (tobacco etch virus) cleavable sequence. *E. coli* BL21(DE3) cells transformed with the pPROEXHtb-EcEFTu plasmid were grown to OD_{600} of 0.6–0.8 at 37

°C, induced with 0.2 mol dm^{-3} IPTG and allowed to grow for 3 h. Overexpressed N-His_6-EF-Tu was purified by a standard procedure employing affinity chromatography on Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) resin. Briefly, the cells were lysed by sonication in buffer containing 50 mmol dm^{-3} Hepes-KOH (pH = 7.5), 10 mmol dm^{-3} $MgCl_2$, 0.5 mol dm^{-3} NaCl, glycerol (φ = 5 %), 100 μmol dm^{-3} GDP, 5 mmol dm^{-3} β-mercaptoethanol and 10 mmol dm^{-3} imidazole. The lysate was cleared by centrifugation, followed by filtration through a 0.22 μm cellulose acetate filter. The filtrate was loaded onto a 1 mL Ni^{2+}-NTA resin and washed with 30 column volumes of lysis buffer, followed by two more stringent washes, each with 10 column volumes of the buffer containing a higher imidazole concentration (20 mmol dm^{-3} and 30 mmol dm^{-3}, respectively). N-His_6-EF-Tu:GDP was eluted in the buffer with 200 mmol dm^{-3} imidazole. Fractions that contained N-His_6-EF-Tu:GDP were pooled, concentrated and dialyzed against 50 mmol dm^{-3} Hepes-KOH (pH = 7.5), 10 mmol dm^{-3} $MgCl_2$, 50 mmol dm^{-3} KCl, glycerol (φ = 5 %), 50 μmol dm^{-3} GDP and 5 mmol dm^{-3} β-mercaptoethanol. Glycerol was added to final φ = 50 % and EF-Tu:GDP was stored at −20 °C. Its purity was determined to be greater than 95 % by SDS-PAGE.

To eliminate traces of endogenous *E. coli* LeuRS, an additional purification step by size-exclusion chromatography was performed using a Superdex 200 HR 10/30 column (GE Healthcare) equilibrated in 50 mmol dm^{-3} Hepes-KOH (pH = 7.5), 10 mmol dm^{-3} $MgCl_2$, 150 mmol dm^{-3} NaCl, glycerol (φ = 5 %), 50 μmol dm^{-3} GDP and 5 mmol dm^{-3} β-mercaptoethanol. Fractions that were enriched in EF-Tu:GDP were pooled and stored as previously described (see above).

Overexpression and purification of TEV protease

A plasmid containing the gene for His-tagged TEV protease was a generous gift from EMBL Protein Expression and Purification Core Facility. *E. coli* Rosetta cells transformed with the pET24-TEV plasmid were grown to OD_{600} 0.6–0.8 at 37 °C. The culture was cooled to 15 °C before adding 0.5 mmol dm^{-3} IPTG, and the cells were allowed to grow for 15 more hours at 15 °C. Cell lysis was performed by sonication in buffer containing 50 mmol dm^{-3} Tris-HCl (pH = 7.5), 300 mmol dm^{-3} NaCl, glycerol (φ = 10 %), 0.2 % (v / v) NP-40 and 10 mmol dm^{-3} β-mercaptoethanol. The lysate was cleared by centrifugation and filtration prior to loading on Ni^{2+}-NTA resin. Chromatography was performed as described for EF-Tu. Fractions enriched with TEV protease were pooled and concentrated to 5 mg mL^{-1} (precipitation was observed at higher concentrations). TEV protease was dialyzed against 25 mM Tris-HCl (pH = 7.5), 150 mM NaCl, glycerol (φ = 10 %), and 5 mmol dm^{-3} β-mercaptoethanol before storage at −80 °C.

Removal of N-terminal His-tag from N-His_6-EF-Tu recombinant protein

1 mg mL^{-1} N-His_6-EF-Tu:GDP was incubated with 0.2 mg mL^{-1} TEV protease in 50 mmol dm^{-3} Hepes-KOH (pH = 7.5), 10 mmol dm^{-3} $MgCl_2$, 50 mmol dm^{-3} KCl, 1 mmol dm^{-3} DTT and 50 μmol dm^{-3} GDP overnight at 4 °C. SDS-PAGE analysis of the reaction mixture established that the digestion reaction reached around 90 % completion. His_6-TEV protease, uncleaved N-His_6-EF-Tu:GDP and cleaved N-terminal His-tag were removed by purification on Ni^{2+}-NTA resin. The EF-Tu:GDP fraction that did not bind to the resin was pooled, concentrated and dialyzed against storage buffer (see above). Glycerol was added to φ = 50 % and EF-Tu:GDP was stored at −20 °C.

Preparation of LeuRS, $tRNA^{Leu}$ and aa-$tRNA^{Leu}$

Wild-type *E. coli* LeuRS and the D345A LeuRS variant defective in hydrolysis of aa-$tRNA^{Leu}$ were overexpressed and purified by affinity chromatography on Ni^{2+}-NTA resin, as described.[19,27] Because LeuRS copurifies with leucyl-adenylate bound in the active site, a

second purification step was performed to ensure its removal.[19] Removal of leucyl-adenylate from the LeuRS active site is essential for preparative $tRNA^{Leu}$ misacylation.

The *E. coli* $tRNA^{Leu}_{TAA}$ isoacceptor was prepared by *in vivo* overexpression and purified as described.[19,27] Radiolabelling of the 3' internucleotide linkage of tRNA with ^{32}P ([^{32}P]-tRNA) was performed by a standard procedure using tRNA nucleotidyl-transferase.[28,29] Leu-[^{32}P]-$tRNA^{Leu}$ and Nva-[^{32}P]-$tRNA^{Leu}$ were prepared by a previously published procedure.[19] Briefly, approximately 0.5 μmol dm^{-3} [^{32}P]-$tRNA^{Leu}$ and 1 μmol dm^{-3} D345A LeuRS were mixed in the standard LeuRS aminoacylation buffer. After approximately 35 min at 37 °C, $tRNA^{Leu}$ was recovered by phenol extraction, desalted on a P30-column (Micro Bio-Spin) and dialyzed against 15 mmol dm^{-3} sodium acetate (pH = 5.0) prior to storage at −20 °C. The fraction of aminoacylated $tRNA^{Leu}$ was established through P1 nuclease digestion and thin-layer chromatography (TLC) analysis.[19,27] aa-tRNA was quantitated from the aa-Ap/(aa-Ap + Ap) ratio, where aa-Ap represents aminoacylated tRNA and Ap represents non-aminoacylated tRNA.

Leu-[^{32}P]-$tRNA^{Leu}$ used to determine the fraction of active EF-Tu (see below), was prepared in a slightly different manner, since that assay requires use of a higher aa-tRNA concentration. 30 μmol dm^{-3} $tRNA^{Leu}$ and 10 μmol dm^{-3} D345A LeuRS were mixed with roughly 200 nmol dm^{-3} [^{32}P]-$tRNA^{Leu}$ in the standard LeuRS aminoacylation buffer. After approximately 45 min at 37 °C, $tRNA^{Leu}$ was recovered by phenol extraction and ethanol precipitation. The pellet was dissolved in 50 mmol dm^{-3} sodium acetate (pH = 5.0), applied to a P30-column (Micro Bio-Spin) and dialyzed against 15 mmol dm^{-3} sodium acetate (pH = 5.0) before storage at −20 °C. The final concentration of aa-$tRNA^{Leu}$ was determined as described.[19]

EF-Tu:GDP activation

Because EF-Tu is purified and stored as the GDP-bound form, it is necessary to convert the protein to the EF-Tu:GTP form before use. Activation of 15 μmol dm^{-3} EF-Tu:GDP was performed in 70 mmol dm^{-3} Hepes-KOH (pH = 7.5), 52 mmol dm^{-3} ammonium acetate, 8 mmol dm^{-3} magnesium acetate, 30 mmol dm^{-3} KCl, 0.8 mmol dm^{-3} DTT, 10 mmol dm^{-3} phosphoenolpyruvate, 1 mmol dm^{-3} GTP and 0.08 U μL^{-1} pyruvate kinase at 37 °C for 2 hours. EF-Tu:GTP was used immediately after the activation procedure.

Determination of the fraction of active EF-Tu:GTP

Only a small fraction of the GTP-bound form of EF-Tu is able to bind aa-tRNA.[30] The fraction of EF-Tu:GTP active in aa-tRNA binding is generally determined by an RNase protection assay[3,4,22] that relies on the ability of EF-Tu:GTP to protect bound aa-tRNA from RNase digestion. The assay was modified in this work to allow TLC separation of digested and non-digested tRNAs. Varying amounts of EF-Tu:GTP (0–12.5 μmol dm^{-3}, total protein concentration) in the activation buffer were mixed with saturating amounts of Leu-[^{32}P]-$tRNA^{Leu}$ (600 nmol dm^{-3}) for 20 min at 4 °C to allow for ternary complex formation. 3 μL of 10 mg mL^{-1} RNase A were then added to the 30 μL reaction mixture to digest free (unbound) aa-tRNA. 2 μL of reaction mixture were taken at several time points, and were quenched in 4 μL of 1.5 mol dm^{-3} formic acid to inactivate RNase A. 2–3 μL of this mixture were then spotted onto polyethyleneimine-cellulose plates (Fluka) prewashed in water. Separation of digested from protected aa-[^{32}P]-tRNA was performed by TLC in 0.1 mol dm^{-3} ammonium acetate and acetic acid (φ = 5 %), followed by quantitation by phosphorimaging. The percentage of aa-[^{32}P]-tRNA protected from RNase A as a function of time was fit to a single exponential equation, and the fraction of aa-[^{32}P]-tRNA initially bound to EF-Tu:GTP was determined from extrapolation to $t = 0$ (time of RNase A addition). The aa-tRNA fraction (bound at $t = 0$) was plotted against the total EF-Tu

concentration, and the fraction of EF-Tu:GTP molecules capable of binding aa-tRNA was determined from the slope of the linear portion of the plot.[30] About 10–15 % of total activated EF-Tu was found to be active in aa-tRNA binding. Throughout this paper, concentrations of EF-Tu:GTP refer to the concentrations of protein capable of aa-tRNA binding, unless otherwise stated.

Control experiments in the absence of EF-Tu were performed to correct for the free aa-tRNA that was not digested within 15 s (first time point) after RNase A addition. Typically, more than 95 % of aa-tRNA was immediately digested and the percentage did not change over time. The remaining aa-tRNA background was subtracted from all experimental data. Control reactions that were performed with varying concentrations of EF-Tu:GDP resulted in immediate digestion of more than 95 % aa-tRNA and matched the reactions performed in the absence of EF-Tu.

Determination of equilibrium dissociation constants (K_D) at 4 °C

Ternary complex formation was monitored by the modified RNase protection assay as described above. Briefly, subsaturating (1–5 nmol dm^{-3}) amounts of aa-[^{32}P]-tRNA were mixed with EF-Tu:GTP and preincubated for 20 min at 4 °C before addition of RNase A. Concentrations of active EF-Tu:GTP were varied in a broad range (5–1400 nmol dm^{-3}) to accurately determine K_D. The fraction of protected aa-[^{32}P]-tRNA was plotted against the concentration of active EF-Tu:GTP and the data were fit to the hyperbolic equation $y = Y_0 \times$ [EF-Tu:GTP]/(K_D+ [EF-Tu:GTP]) where Y_0 is the maximal protected fraction and K_D is the dissociation constant.

Determination of dissociation rate constants (k_{off}) at 4 °C

The EF-Tu:GTP:aa-tRNA complex was formed by mixing 600 nmol dm^{-3} aa-[^{32}P]-tRNA and approximately 1.5 μmol dm^{-3} active EF-Tu:GTP in EF-Tu activation buffer. The stability of the complex was monitored by the modified RNase protection assay. RNase A was added after a 20 min equilibration period at 4 °C, and time points were collected in a range from 0.15–15 min by mixing 2 μL of reaction mixture with 4 μL of 1.5 mol dm^{-3} formic acid, followed by TLC analysis as described above. The fraction of aa-[^{32}P]-tRNA protected from RNase A was fit to the single exponential equation $y = Y_0 + A \times e^{-k\text{off} \times t}$ where Y_0 is the y intercept, A is the amplitude, k_{off} is the observed dissociation rate constant and t is time.

Determination of equilibrium dissociation constants (K_D) at 37 °C

The assay is based on measuring the protective effect of a EF-Tu:GTP:aa-tRNA ternary complex on the non-enzymatic deacylation of aa-tRNA.[23,24] The reactions were performed by incubating EF-Tu:GTP with aa-[^{32}P]-tRNA^{Leu} at 37 °C in the activation buffer. aa-[^{32}P]-tRNA^{Leu} was present at 5–10 nmol dm^{-3} concentration and the concentration of EF-Tu:GTP was varied over a wide range (30–1700 nmol dm^{-3}) to most accurately determine K_D. Reactions were stopped at different time points by mixing 2 μL aliquots of reaction mixture with 4 μL of quench solution containing 0.75 mol dm^{-3} sodium acetate (pH = 4.5) and 1.5 g dm^{-3} SDS. The fraction of aa-tRNA in each time point was determined through P1 nuclease digestion and analysis on TLC plates.[19] Data were fit to the single exponential equation $y = A \times e^{-k\text{obs} \times t}$ where A is the amplitude, k_{obs} is the observed non-enzymatic deacylation rate constant, and t is time. Non-enzymatic deacylation rate constants were plotted against concentration of the active EF-Tu:GTP and fit to the equation $k_{obs} = k_{unprotected}/(1 +$ [EF-Tu:GTP]/K_D), where $k_{unprotected}$ is the observed constant for non-enzymatic deacylation rate of aa-tRNA in the absence of EF-Tu:GTP and K_D represents the dissociation constant of EF-Tu:GTP:aa-tRNA ternary complex.[24] The specificity of the interaction was verified by

control reactions performed with several concentrations of EF-Tu:GDP, where the presence of inactive EF-Tu had no protective effect on non-enzymatic deacylation.

RESULTS

Preparation of EF-Tu suitable for use in experiments with Nva-tRNA^{Leu}

Here we study interactions of EF-Tu with Leu-tRNA^{Leu} and Nva-tRNA^{Leu} using modified versions of the ribonuclease protection and non-enzymatic hydrolysis protection assays.[3,4,22–24] For both assays, high sensitivity to even small contamination by endogenous LeuRS was expected, because (i) low levels of aa-[^{32}P]-tRNA were employed (because of the much higher sensitivity as compared with [^{14}C]-aa-tRNA) and (ii) Nva-tRNA^{Leu}, an efficient (natural) substrate for hydrolytic clearance by LeuRS,[19] was used. Further, EF-Tu:aa-tRNA interactions are generally studied using high EF-Tu (total protein) concentrations, because only a small fraction of EF-Tu:GTP molecules are active in aa-tRNA binding.[30] This also makes analysis sensitive to contaminations in the protein sample. To test for the presence of endogenous *E. coli* LeuRS, the EF-Tu:GDP was tested for Leu-tRNA^{Leu} formation in the standard aminoacylation assay. Significant aminoacylation activity was observed with 12.5 μmol dm^{-3} EF-Tu:GDP (total protein concentration); comparison with aminoacylation rate achieved by 2 nmol dm^{-3} LeuRS indicates a contamination level of approximately 0.005 % (Figure 1B).

Next, EF-Tu:GDP was converted to EF-Tu:GTP and tested for interaction with Nva-tRNA^{Leu} using the non-enzymatic hydrolysis protection assay (see below). Significant hydrolysis of Nva-tRNA^{Leu} instead of protection was observed (Figure 1C), confirming that even low levels of copurified LeuRS preclude determination of EF-Tu:Nva-tRNA^{Leu} affinity. To remove these traces of LeuRS, EF-Tu:GDP sample was additionally purified by size-exclusion chromatography (see Experimental section). After this purification, EF-Tu:GDP showed no detectable leucylation activity (Figure 1B) and when activated to EF-Tu:GTP, it efficiently protected Nva-tRNA^{Leu} from non-enzymatic deacylation (Figure 1C). This demonstrates that endogenous LeuRS was completely removed by this additional purification step.

The modified ribonuclease protection assay

The ribonuclease protection assay[3,4,22] relies on the ability of EF-Tu to protect aa-tRNA from RNase A digestion, thus distinguishing between the bound and free aa-tRNA ligand. The free aa-tRNA is rapidly hydrolyzed by RNase A during a short incubation period, while the bound aa-tRNA remains protected. [^{14}C]-amino acid is generally used to label the aa-tRNA, and digested and protected tRNAs are distinguished by their acid-solubility or acid-insolubility, respectively. Thus, the fraction of aa-tRNA bound to EF-Tu (and thus protected from RNase A digestion) is commonly determined from the radioactivity present in the acid precipitates. Here, we present a modified version of this assay, where the different behavior of digested and protected aa-tRNAs in thin-layer chromatography, instead of different acid-solubilities, are used for separation (Figure 2). The main advantage of this approach is that it is not complicated by the precipitation and filtering steps, and can be easily used in a high-throughput format requiring only a multichannel pipette and 96 well plates. We also used [^{32}P]-tRNA (labeled at the terminal adenosine using tRNA nucleotidyl-transferase)[28,29] to produce aa-[^{32}P]-tRNAs. Use of radiolabeled tRNA was obligatory for studying interactions of EF-Tu with Nva-tRNA^{Leu} because [^{14}C]-Nva is not commercially available.

We first incubated aa-[^{32}P]-tRNA with RNase A, at the same concentrations used in the ribonuclease protection assay, to establish the chromatographic pattern resulting from digestion (Figure 2A). tRNA used in all assays was aminoacylated up to 50–60 % by either

leucine or norvaline. Incomplete aminoacylation is not consequential, because the presence of non-aminoacylated tRNA does not influence EF-Tu binding affinity for aa-tRNA.[9] Indeed, tRNA samples with less than 30 % of aminoacylated tRNA have previously been successfully used.[9] tRNA is rapidly hydrolyzed within 15 s (Figure 2A), confirming that the amount of RNase A is sufficient for rapid and complete digestion. We have also observed a substantial change in chromatographic mobility that allows separate quantitation of digested and non-digested tRNAs. In agreement with previous findings,[30] about 5 % of tRNA remained non-digested or was digested in a way that does not influence its TLC mobility (Figure 2A). This value was subtracted as background from all quantitated data. To perform reliable time-dependent measurements, very rapid inactivation of RNase A digestion is required before TLC analysis. We tested formic acid as a possible quench by preincubating aa-tRNA in 1 mol dm^{-3} formic acid prior to addition of RNase A. As observed from Figure 2A, 98 % of tRNA was not digested within 30 s or 30 min under these conditions. Thus, RNase A is rapidly inactivated in 1 mol dm^{-3} formic acid, making it a suitable quench reagent for the RNase A reaction.

EF-Tu:GTP binds Nva-tRNA^{Leu} and Leu-tRNA^{Leu} with a similar affinity

We first used the modified ribonuclease protection assay to test if EF-Tu discriminates between Leu-tRNA^{Leu} and Nva-tRNA^{Leu}. We used this assay to extract both equilibrium (K_D) and rate (k_{on}, k_{off}) constants describing the interactions between *E. coli* EF-Tu and either Leu-tRNA^{Leu} or Nva-tRNA^{Leu}, as previously described by Uhlenbeck and colleagues (4,5,9).

For equilibrium measurements, various concentrations of EF-Tu:GTP (EF-Tu:GDP was converted to EF-Tu:GTP immediately prior to use) were incubated for 25 min at 4 °C with either Leu-[^{32}P]-tRNA^{Leu} or Nva-[^{32}P]-tRNA^{Leu}. Bound and unbound aa-[^{32}P]-tRNA were distinguished by short incubation with RNase A, where only unbound aa-tRNA has been digested, followed by quenching in formic acid (to inactivate RNase) and separation from the bound aa-tRNA by TLC (for details see Experimental). Digested and protected tRNAs (representing unbound and bound tRNAs, respectively) were independently quantitated (Figure 2B), and the fraction of bound aa-tRNA was calculated and plotted against the concentration of active EF-Tu:GTP (for determination of the fraction of active EF-Tu:GTP see Experimental section) (Figure 3A). Our data show that EF-Tu:GTP binds Leu-tRNA^{Leu} and Nva-tRNA^{Leu} with similar affinity (70 nmol dm^{-3} and 24 nmol dm^{-3}, respectively, Figure 3A and Table 1). To the best of our knowledge, these are the first data showing interaction of EF-Tu with norvalylated tRNA. The similar affinities measured for Leu-tRNA^{Leu} and Nva-tRNA^{Leu} are consistent with the data showing incorporation of norvaline in cellular proteins under some conditions.[14,15]

Next, we determined the dissociation rate constants of Leu-tRNA^{Leu} and Nva-tRNA^{Leu} (k_{off}) from their corresponding EF-Tu:GTP:aa-tRNA ternary complexes using the modified version of ribonuclease protection assay. The rationale was to examine if kinetics of association (k_{on} and k_{off}) significantly differs for these two aa-tRNAs, in spite of the similar overall K_D. EF-Tu:GTP and aa-tRNA were incubated for 25 min at 4 °C followed by RNase A addition. Time points were taken at regular intervals, and the reaction was quenched in 1 mol dm^{-3} formic acid. Digested and protected tRNAs were separated as described for the equilibrium measurements (Figure 2). The fraction of remaining bound aa-tRNA was plotted *versus* time, and first order rate constant representing k_{off} was extracted (Figure 3B). Similar to our findings with respect to equilibrium constants, the dissociation rate constants (k_{off}) for Leu-tRNA^{Leu} and Nva-tRNA^{Leu} are also highly similar (Figure 3A and Table 1). Taken together, our data clearly show that neither equilibrium binding nor association kinetics differ significantly between EF-Tu:GTP:Leu-tRNA^{Leu} and EF-Tu:GTP:Nva-tRNA^{Leu}

ternary complexes. Thus, *E. coli* EF-Tu does not distinguish between Leu-tRNA^{Leu} and Nva-tRNA^{Leu}.

Discrimination of norvaline by EF-Tu:GTP is not enhanced at the physiologically relevant temperature

The data so far presented describes the EF-Tu:GTP:aa-tRNA interactions at 4 °C, a temperature that is not physiologically highly relevant, but is commonly used in EF-Tu binding studies. Two main advantages of working at lower temperatures are (i) higher affinity of EF-Tu:GTP for aa-tRNA and (ii) significantly slower aa-tRNA dissociation rate allowing manual sampling of time points.[4,31] However, there is a possibility that EF-Tu:GTP discriminates better against Nva-tRNA^{Leu} at the physiologically more relevant temperature. To experimentally address this issue, we used a different assay,[23,24] better suited for the work at higher temperatures. This assay is based on protection of aa-tRNA from non-enzymatic hydrolysis in solution, when it is bound to EF-Tu:GTP. The GDP-bound form of EF-Tu was firstly converted to the GTP-bound form prior to mixing with aa-[^{32}P]-tRNA at 37 °C. At certain time points, reaction aliquots were quenched, tRNA was degraded using P1 nuclease, and products were analyzed by TLC as described.[19,27] Representative time courses obtained at different concentrations of active EF-Tu:GTP are shown in Figure 4A. As expected, at higher concentrations of EF-Tu:GTP, the pre-equilibrium is shifted toward preferential binding of aa-tRNA, thus decreasing the rate of non-enzymatic hydrolysis (*i.e.* better protection is observed). As a control, the same experiment was performed with EF-Tu:GDP (Figure 4B). No protection of Leu-tRNA^{Leu} was observed, confirming the specificity of interactions measured in Figure 4A. The dependence of k_{obs} *versus* concentration of the active EF-Tu:GTP yields K_D (Figure 4C and Table 1). Interestingly, a similar level of discrimination is observed at 37 °C and 4 °C, which further demonstrates that Nva-tRNA^{Leu} and Leu-tRNA^{Leu} are equally good substrates for EF-Tu:GTP-mediated transport to the ribosome.

DISCUSSION

Quality control of protein translation is manifested at several steps: formation of aa-tRNAs by aaRSs, EF-Tu:GTP dependent delivery of aa-tRNAs to the ribosome, and the subsequent mRNA decoding, where the anticodon of aa-tRNA is matched with the cognate codon to ensure incorporation of the esterified amino acid at the appropriate position in the growing polypeptide chain. Each of these steps possesses an inherent error frequency and mechanism to maintain error rates within the level tolerable in protein synthesis (10^{-3}–10^{-4}).[16] Prompted by the findings that the non-proteinogenic amino acid norvaline partially evades translational proofreading mechanisms, and is thus incorporated into proteins in place of leucine under some conditions *in vivo*,[14,15] we have recently analyzed in detail the capacity of *E. coli* LeuRS to discriminate against formation of Nva-tRNA^{Leu}.[19] In agreement with previous findings,[18] norvaline was indeed determined to be a reasonably good substrate for the LeuRS synthetic reactions. However, accumulation of Nva-tRNA^{Leu} was not observed due to its rapid hydrolysis (the single turnover rate constant is 300 s^{-1}) within the LeuRS CP1 editing site.[19] Here, we further explore the capacity of Nva-tRNA^{Leu} to bind EF-Tu:GTP. The rationale was to determine whether any Nva-tRNA^{Leu} that evades LeuRS hydrolytic editing may participate efficiently in the subsequent step in translation.

First, we tested equilibrium binding of Nva-tRNA^{Leu} and Leu-tRNA^{Leu} to EF-Tu:GTP at 4 °C using a modified version of the commonly used ribonuclease protection assay to extract K_D values. Previous work has shown that all elongator aa-tRNAs bind *E. coli* EF-Tu:GTP similarly, with only a 12-fold range in the K_D values.[3] Thus, the 3-fold difference in K_D values for Nva-tRNA^{Leu} and Leu-tRNA^{Leu} (Table 1) strongly suggests that Nva-tRNA^{Leu} is indistinguishable from Leu-tRNA^{Leu} and other elongator cognate aa-tRNAs regarding its

interaction with EF-Tu. Moreover, the observed K_D values are very similar to the previously determined K_D for *E. coli* Phe-tRNA^{Phe} under comparable ionic strength conditions.[32] Analysis of association kinetics revealed the same pattern. k_{off} for Nva-tRNA^{Leu} and Leu-tRNA^{Leu} differs by less than 2-fold (Table 1) and the values are highly similar to the previously determined k_{off} for Phe-tRNA^{Phe}.[31,32] In general, EF-Tu K_D values are less accurate than k_{off} values because of the error-prone determination of the active EF-Tu:GTP fraction, which is a prerequisite for K_D extraction (Figure 3). Because k_{on} was shown to be constant for different aa-tRNAs,[32] K_D is often calculated from the k_{on} value[32] (1.1×10^5 mol^{-1} dm^3 s^{-1}) and the experimentally determined k_{off}.[5,12,30] Here, we calculated k_{on} from the experimentally measured K_D and k_{off} values for both Leu-tRNA^{Leu} and Nva-tRNA^{Leu}. The values obtained (Table 1) are very similar to each other and to the previously determined k_{on} value. This provides considerable confidence in the reported thermodynamic and kinetic parameters and strongly supports the conclusion that Nva-tRNA^{Leu} is not discriminated in translation at the level of EF-Tu:GTP binding.

Additional proof was obtained from the analysis of EF-Tu:GTP interactions with both Nva-tRNA^{Leu} and Leu-tRNA^{Leu} at the physiologically more relevant temperature of 37 °C. Here we used an assay that relies on protection of aa-tRNA from solution-based non-enzymatic hydrolysis when bound to EF-Tu:GTP.[23,24] Again, the extracted K_D values for Nva-tRNA^{Leu} and Leu-tRNA^{Leu} (Table 1) were highly similar, demonstrating that discrimination against norvaline is independent of temperature. We also show that aa-tRNA binds EF-Tu:GTP weaker at higher temperatures, consistent with previous observations.[4,31] The absence or presence of the N-terminal His_6-tag on EF-Tu did not influence its interactions with aa-tRNAs (Table 1).

According to the thermodynamic compensation model,[4] the contributions of the esterified amino acid and the tRNA body are independent of one another, but compensate such that all cognate aa-tRNA pairs have similar binding affinities. Because fine tuning of binding affinities by the compensation mechanism is disturbed in misacylated tRNAs, these species bind EF-Tu:GTP over a broad range of affinities, varying from 60-fold weaker to 120-fold tighter as compared with cognate aa-tRNA pairs.[5] This apparently depresses utilization of at least some misacylated tRNAs in translation, and thus EF-Tu may act as a checkpoint for translational fidelity.[10–12] Structural[33,34] and thermodynamic analyses[5] revealed that amino acids bind in the same pocket on the surface of EF-Tu, but establish slightly different contacts that result in different binding affinities. Lack of discrimination against Nva-tRNA^{Leu} suggests that both norvaline and leucine establish interactions with EF-Tu:GTP that are thermodynamically comparable. Inspection of *Thermus thermophilus* EF-Tu interactions with specifically designed misacylated tRNAs revealed that valine binds approximately 2-fold weaker than the slightly bigger isoleucine.[5] Comparison of the measured k_{off} values (Table 1) revealed that norvaline, although smaller, binds EF-Tu approximately 1.5-fold tighter than leucine. Thus, it is likely that norvaline compensates for the lack of the methyl group binding energy by establishing better interactions of its unbranched side chain within the EF-Tu binding pocket.

Our extensive kinetic analysis demonstrated that wild-type LeuRS very efficiently clears Nva-tRNA^{Leu} *in vitro*.[19] However, anything that disturbs the kinetic partitioning of Nva-tRNA^{Leu} between post-transfer editing hydrolysis and dissociation (either *via* compromising hydrolytic activity or stimulating dissociation) may result in accumulation of Nva-tRNA^{Leu}. It is not yet understood what factors may influence kinetic partitioning of Nva-tRNA^{Leu} *in vivo*. However, what we clearly established here is that after Nva-tRNA^{Leu} is released from LeuRS, it binds EF-Tu as efficiently as Leu-tRNA^{Leu} or any other elongator aa-tRNA. This explains the occurrence of norvaline incorporation in place of leucine *in vivo*, under conditions where its accumulation allows efficient activation by LeuRS.[14,15] Taken together,

these findings strongly suggest that hydrolytic editing by LeuRS serves as the main quality control checkpoint against incorporation of norvaline into cellular proteins. Indeed, when LeuRS hydrolytic editing was compromised, a significant substitution of leucine by norvaline was observed in a reporter protein *in vivo*.[18] Similarly, mistranslation of phenylalanine codons by tyrosine parallels the utilization of an editing-deficient phenylalanyl-tRNA synthetase (PheRS) in poly(U)-directed polyTyr/polyPhe synthesis assay. In this case it was shown that mistranslation occurs in part from the inability of *E. coli* EF-Tu to discriminate between Phe-tRNA^{Phe} and Tyr-tRNA^{Phe}.[31] One may argue that because of efficient editing by aaRSs, such as PheRS[35,36] and LeuRS,[19,37,38] EF-Tu was not subject to evolutionary pressure to develop stringent discrimination against Tyr-tRNA^{Phe} and Nva-tRNA^{Leu}. Thus, EF-Tu may have preferentially evolved towards efficient elimination of commonly occurring mischarged intermediates such as Glu-tRNA^{Gln} and Asp-tRNA^{Asn}.[4,9–11,24] The failure of EF-Tu to discriminate among some aminocyl-tRNA substrates places the main burden of translational fidelity on the corresponding aaRSs. It appears that the interplay between attainable accuracies in cognate aa-tRNA formation and EF-Tu recognition may have driven evolution, at least in the case of LeuRS, towards acquisition of the highly efficient hydrolytic site that prevents accumulation of Nva-tRNA^{Leu} for mistranslation.

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The abbreviations used are

aaRS	aminoacyl-tRNA synthetase
aa-tRNA	aminoacyl-tRNA
CP1	connective peptide 1
EF-Tu	elongation factor Tu
LeuRS	leucyl-tRNA synthetase
Nva	norvaline
PheRS	phenylalanyl-tRNA synthetase
RNase	ribonuclease
TEV	tobacco etch virus
TLC	thin-layer chromatography
WT	wild-type.

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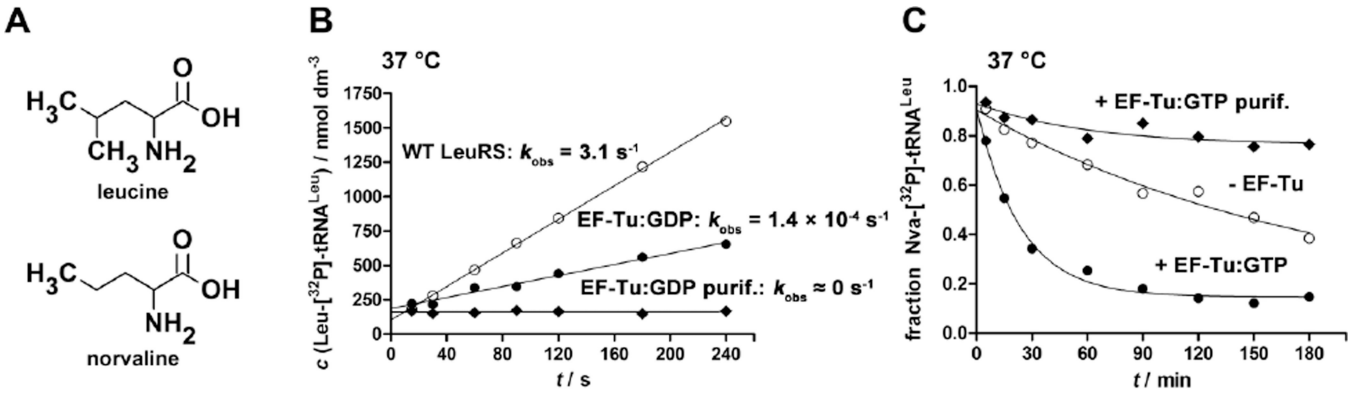

Figure 1.

E. coli EF-Tu prepared by the standard Ni^{2+}-NTA purification procedure contains traces of copurified endogenous *E. coli* LeuRS. *A*, molecular structures of leucine and norvaline. *B*, representative leucylation time course with 12.5 μmol dm^{-3} EF-Tu:GDP before (●) and after (♦) additional purification by size-exclusion chromatography. The amount of LeuRS that contaminates EF-Tu was estimated by comparing leucylation rates of 12.5 μmol dm^{-3} EF-Tu (total protein concentration) and 2 nmol dm^{-3} LeuRS (○). *C*, representative Nva-[^{32}P]-$tRNA^{Leu}$ deacylation time courses in the absence of EF-Tu (○) and in the presence of activated (●) or purified and activated EF-Tu ((♦).

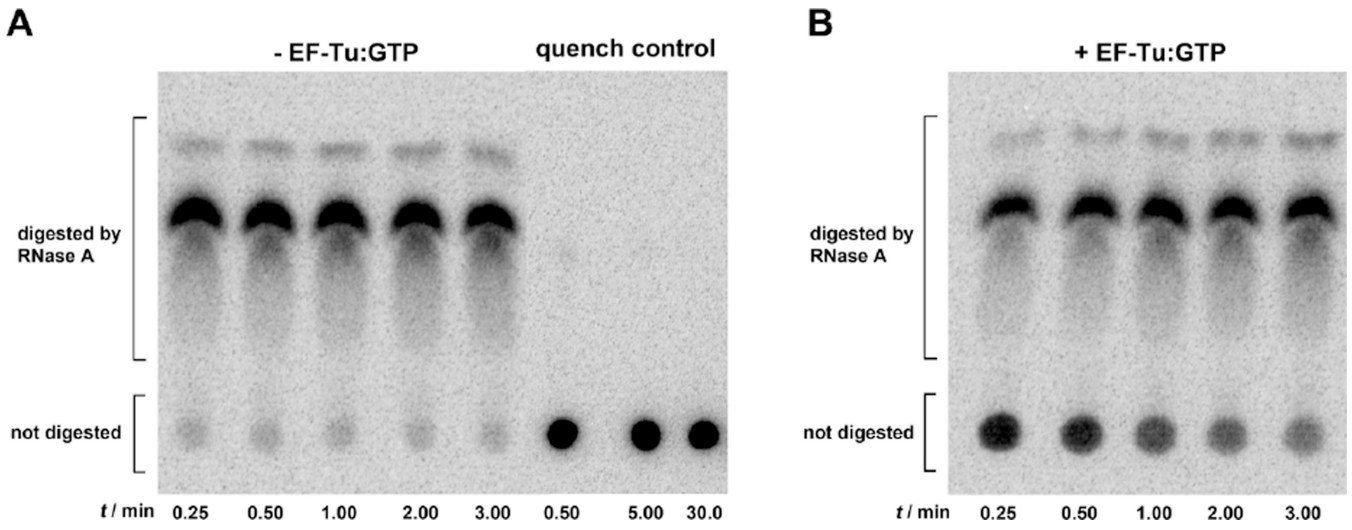

Figure 2.

Characteristic RNase digestion pattern obtained by thin-layer chromatography. *A*, the thin-layer chromatogram represents a time course obtained by incubating Leu-[^{32}P]-tRNA^{Leu} with RNase A in the absence of EF-Tu:GTP (left side of the panel, -EF-Tu:GTP) and a quench control performed by adding RNase A to Leu-[^{32}P]-tRNA^{Leu} mixed with 1 mol dm^{-3} formic acid (right side of the panel, quench control). Digested and non-digested tRNAs were separately quantitated with ImageQuant software and the fraction of non-digested tRNA was calculated by dividing the intensity of non-digested tRNA with the total intensity. To calculate the fraction of non-digested aa-tRNA, the fraction of non-digested tRNA was divided by the fraction of aa-tRNA initially present in the sample. *B*, representative thin-layer chromatogram of a time course obtained in the modified RNase protection assay where RNase A was added to a mixture of Leu-[^{32}P]-tRNA^{Leu} and EF-Tu:GTP preincubated at 4 °C.

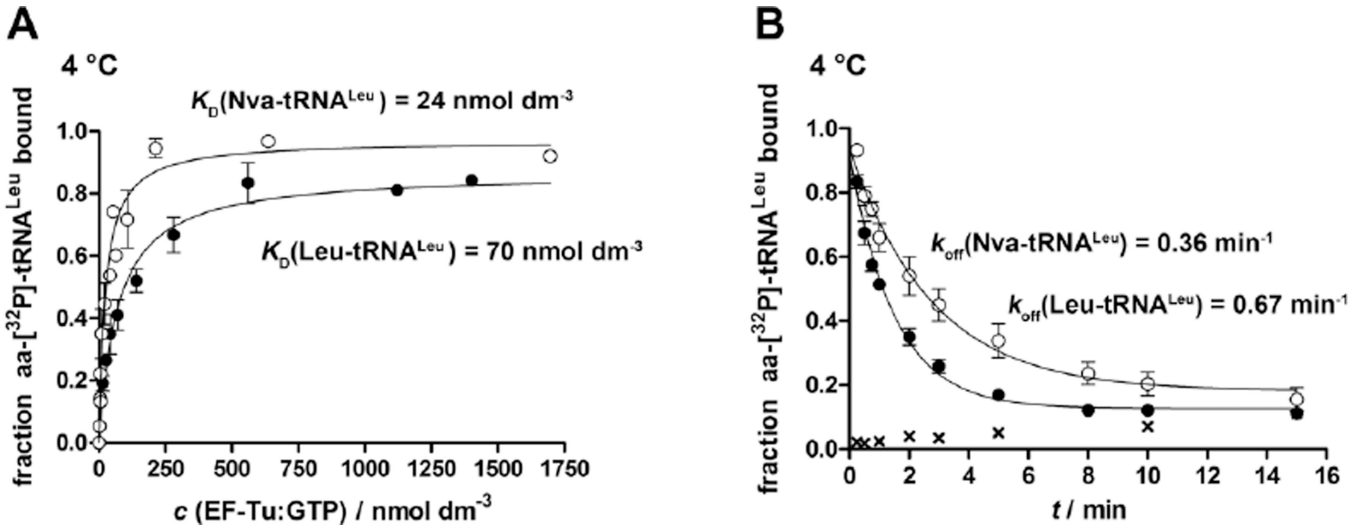

Figure 3.

Interaction of *E. coli* EF-Tu:GTP with Leu- or Nva-[^{32}P]-tRNA^{Leu} at 4 °C. *A*, equilibrium binding curves showing affinity of *E. coli* EF-Tu:GTP for Leu-tRNA^{Leu} (●) or Nva-tRNA^{Leu} (○). *B*, time courses depicting Leu-tRNA^{Leu} (●) or Nva-tRNA^{Leu} (○) dissociation from aa-tRNA^{Leu}:EF-Tu:GTP ternary complex. The control experiment was performed with EF-Tu:GDP (×).

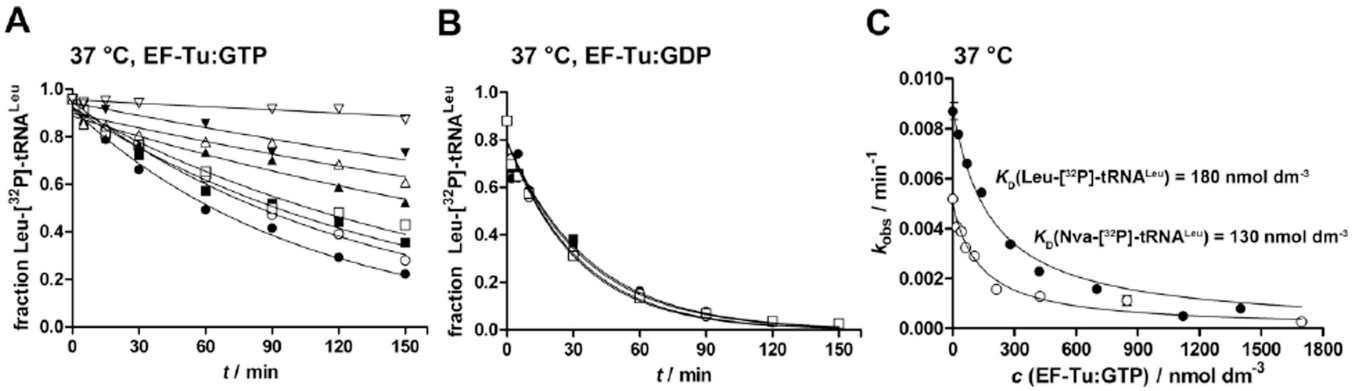

Figure 4.

Interaction of *E. coli* EF-Tu:GTP with Leu- or Nva-[^{32}P]-tRNA^{Leu} at 37 °C. *A*, representative Leu-[^{32}P]-tRNA^{Leu} non-enzymatic deacylation time courses in the presence of 0 (●), 28 (○), 70 (■), 140 (□), 280 (▲), 420 (△), 700 (▼) and 1120 (▽) nmol dm^{-3} active EF-Tu:GTP. *B*, control non-enzymatic Leu-[^{32}P]-tRNA^{Leu} deacylation time courses in the presence of 0 (●), 1 (○), 5 (■) and 10 (□) μmol dm^{-3} EF-Tu:GDP (total protein concentration). *C*, equilibrium binding curves showing affinity of *E.coli* EF-Tu:GTP for Leu-tRNA^{Leu} (●) or Nva-tRNA^{Leu} (○).

Table 1

Thermodynamic and kinetic parameters describing EF-Tu:GTP:aa-tRNA interactions

aa-tRNA^{Leu}	K_D (4 °C)	k_{off} (4 °C)	k_{on} (4 °C) calculated[(a)]	K_D (37 °C)
	nmol dm^{-3}	min^{-1}	mol^{-1} dm^3 min^{-1}	nmol dm^{-3}
Leu-tRNA^{Leu}	70 ± 9	0.67 ± 0.05[(b)]	9.5×10^6	(18 ± 2) × 10[(c)]
Nva-tRNA^{Leu}	24 ± 4	0.36 ± 0.05	15×10^6	(13 ± 1) × 10

The values represent the best fit value ± s.e.m. of three independent experiments.

[(a)] $k_{on} = k_{off}/K_D$

[(b)] 1.3 ± 0.2 min^{-1} determined with N-His$_6$-EF-Tu:GTP

[(c)] (19 ± 2) × 10 nmol dm^{-3} determined with N-His$_6$-EF-Tu:GTP