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Improved Incorporation of Noncanonical Amino Acids by an Engineered tRNA^{Tyr} Suppressor

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Abstract

The *Methanocaldococcus jannaschii* tyrosyl-tRNA synthetase (TyrRS):tRNA^{Tyr} cognate pair has been used to incorporate a large number of noncanonical amino acids (ncAAs) into recombinant proteins in *Escherichia coli*. However, the structural elements of the suppressor tRNA^{Tyr} used in these experiments have not been examined for optimal performance. Here, we evaluate the steady-state kinetic parameters of wild-type *M. jannaschii* TyrRS and an evolved 3-nitrotyrosyl-tRNA synthetase (nitroTyrRS) toward several engineered tRNA^{Tyr} suppressors, and we correlate aminoacylation properties with the efficiency and fidelity of superfolder green fluorescent protein (sfGFP) synthesis *in vivo*. Optimal ncAA-sfGFP synthesis correlates with improved aminoacylation kinetics for a tRNA^{Tyr} amber suppressor with two substitutions in the anticodon loop (G34C/G37A), while four additional mutations in the D and variable loops, present in the tRNA^{Tyr} used in all directed evolution experiments to date, are deleterious to function both *in vivo* and *in vitro*. These findings extend to three of four other evolved TyrRS enzymes that incorporate distinct ncAAs. Suppressor tRNAs elicit decreases in amino acid K_m values for both TyrRS and nitroTyrRS, suggesting that direct anticodon recognition by TyrRS need not be an impediment to superior performance of this orthogonal system and offering insight into novel approaches for directed evolution. The G34C/G37A tRNA^{Tyr} may enhance future incorporation of many ncAAs by engineered TyrRS enzymes.

Graphical Abstract

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Author Contributions

B.J.R. and J. J. Porter contributed equally to this work.

Notes

The authors declare no competing financial interest.

Supporting Information

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Representative V_0 versus S plots for the kinetic data provided in Table 1, structures for the noncanonical amino acids used in the experiments depicted in Figure 5, and information regarding the compositions of autoinducing and noninducing media used in selections (PDF)

Site-specific incorporation of noncanonical amino acids (ncAAs) with genetic code expansion offers substantial potential for the development of new designer proteins for insights into cellular processes^{1–3} and is also a key foundational technology in synthetic biology.⁴ The key requirement for all new coding applications is the design of novel aminoacyl-tRNA synthetases (aaRS) that can ligate one or more ncAAs to a unique tRNA that reads an unused codon triplet or quadruplet.^{5–8} Novel aaRS-tRNA pairs must efficiently function in both aminoacylation and downstream protein synthesis steps *in vivo*, including interactions with elongation factors and the ribosome.

Directed evolution has been used to derive engineered aaRS enzymes capable of introducing >100 ncAAs into proteins in bacterial and eukaryotic cells.^{6,9–11} The first step in the directed evolution process is to modify an aaRS-tRNA pair so that it is orthogonal to the naturally occurring aaRS-tRNA systems in the host cell.⁶ Orthogonality requires that the new aaRS does not attach the ncAA to any endogenous cellular tRNA at rates that would compromise the specificity of protein synthesis *in vivo* or result in heterogeneous incorporation of the ncAA. In addition, proper functioning of the new orthogonal pair demands that an endogenous aaRS does not efficiently aminoacylate the new tRNA with a canonical amino acid.^{9,12} The second step of directed evolution is to alter the active site of the aaRS so that it will accommodate the desired ncAA and attach it to the cognate tRNA while excluding other amino acids.⁶ Although a number of aaRS-tRNA systems have been examined as candidate scaffolds for directed evolution,¹³ two that reliably pass both engineering steps multiple times to incorporate many different ncAAs have emerged: the tyrosyl-tRNA synthetase (TyrRS)-tRNA^{Tyr} complex from *Methanocaldococcus jannaschii*⁹ and the pyrrolysyl-tRNA synthetase (PylRS)-tRNA^{Pyl} pairs from *Methanosarcinae*.¹⁴

Engineered cells incorporating orthogonal aaRS-tRNA pairs are sometimes capable of generating recombinant ncAA-containing proteins at milligram yields per liter of culture,¹⁵ demonstrating that the systems can function very efficiently *in vivo*. Notwithstanding, it is generally recognized that the aspirations of synthetic biology, which include large scale rebuilding of the protein synthesis apparatus to accommodate “designer codes”,¹¹ will require much better integration of the new aaRS-tRNA pairs into the translational apparatus. A central issue is that very high concentrations of ncAAs and over-expression of the new tRNAs are presently needed for recombinant protein expression, but these features may diminish cellular fitness by increasing the level of competition with endogenous aaRS for both amino acid and tRNA pools.^{7,16} Kinetic analysis of several purified PylRS variants that incorporate ncAAs revealed aminoacylation efficiencies reduced by several hundred-fold

compared with that of wild-type PylRS,¹⁷ validating the notion that the directed evolution process does not generate optimally functioning aaRS.

A variety of strategies have been employed to increase recombinant ncAA-containing protein yields while maintaining (i) high levels of fidelity against other amino acids and (ii) low misacylation levels of the new tRNA by host aaRS and of host tRNAs by the evolved aaRS (the orthogonality requirement). For example, recent work to improve expression of noncanonical amino acids in insect and mammalian cells has focused on augmenting suppressor tRNA expression levels, with concomitant increases in yields of the ncAA-containing proteins.^{18–20} Use of a baculovirus-based delivery system to deliver both the engineered tRNA and aaRS components to mammalian cells has also been successful.²¹ Another strategy has been to manipulate interactions with other components of the protein translation system, including release factors in bacterial and mammalian cells,^{18,22} and the elongation factor Tu in bacteria.^{23,24} The orthogonality of *M. jannaschii* tRNA^{Tyr} was improved by overexpression of prolyl-tRNA synthetase (ProRS), outcompeting an undesired interaction of engineered *M. jannaschii* TyrRS with *Escherichia coli* tRNA^{Pro}.²⁵ This exemplifies the long-understood principle that optimal function of the translation system *in vivo* depends on the proper balance of aaRS and tRNA.^{11,26} Finally, directed evolution experiments targeted at the anticodon recognition interface of *M. jannaschii* TyrRS also led to improved incorporation efficiencies of some ncAAs.²⁷

As a contribution to these efforts, we focus here on improving recombinant ncAA-containing protein expression by examining the performance of an orthogonal aaRS:tRNA pair in detail. Recent other work along these lines has studied the PylRS:tRNA^{Pyl} system for ncAA incorporation.^{17,28} Here, we examine modified *M. jannaschii* TyrRS enzymes that insert 3-nitrotyrosine (nitroTyr) and other ncAAs into recombinant proteins *in vivo*.^{29,30} To understand the role of oxidative stress-induced nitration, it is first necessary to express homogeneously nitrated proteins in *E. coli*, which requires an improved efficiency of nitroTyr incorporation.^{29,31,32} Tyrosine nitrosylation is extensive in human cells, is correlated with disease phenotypes, and has been shown to alter protein function *in vivo*.^{33,34} Optimization of 3-nitrotyrosyl tRNA synthetases (nitroTyrRSs) will provide valuable research tools for studying the nitrated human proteome. In prior studies, we established that modifying the experimental protocol for directed evolution, including alterations to the selection media and antibiotic concentrations, yielded new nitroTyrRS variants with markedly different efficiencies for incorporation of nitroTyr into the fluorescent reporter protein sfGFP.³⁰ Improved second-generation enzymes were able to incorporate nitroTyr at multiple sites in Hsp90 and apoA1 at efficiencies markedly superior to those of first-generation enzymes evolved using a more common directed evolution approach.³⁵ The best second-generation nitroTyrRS contains five substitutions, all located directly within the substrate amino acid binding pocket.³⁰

To understand and further improve the function of the nitroTyrRS derived from directed evolution, we measured the kinetic parameters of the best recombinant first- and second-generation nitroTyrRSs as a means of correlating *in vivo* performance in protein synthesis with *in vitro* enzymatic properties. We also examined the role of six nucleotides in *M. jannaschii* tRNA^{Tyr} that were previously mutated to improve orthogonality and that have

been incorporated into the tRNA used in all directed evolution experiments with this orthogonal pair.¹² The data reveal a clear correlation between *in vivo* protein synthesis efficiency and kinetic parameters for aminoacylation over a set of variant enzyme:tRNA pairs. We show that the identity of the nucleotide located immediately 3' to the anticodon sequence plays a key role in modulating incorporation of four different ncAAs *in vivo* and *in vitro*, and that substitution of four other nucleotides in the tRNA^{Tyr} core region is detrimental to protein expression. These findings should provide a basis for improving the performance of many directed evolution systems that employ the *M. jannaschii* TyrRS platform.

EXPERIMENTAL PROCEDURES

Expression Plasmids for Aminoacyl-tRNA Synthetases

To construct *E. coli* expression vectors for *M. jannaschii* TyrRS variants, the DNA fragments containing the variant of interest were amplified by polymerase chain reaction (PCR) from the corresponding pBK plasmids and ligated into the *NcoI/XhoI* sites of expression vector *pET28a(+)*.³⁰ Primers were used to install an N-terminal six-histidine tag (forward primer, 5'-CGCGCGCCATGGACGAATTTGAAATG-3'; reverse primer, 5'-GGGCGCTCGAGTAATCTCTTTCTAATTGGCTCTAAAATC-3'). The resulting *pET-RS* plasmid was transformed into DH10B cells and purified with a QIAprep spin mini kit.

Expression Plasmids for tRNAs

To introduce mutant tRNAs into the pALS plasmid, a DNA fragment containing the altered tRNA sequences was inserted via isothermal assembly.³⁶ The pALS plasmid was amplified by PCR using forward primer 5'-CCACTTATTTTGGATCGTTTCGCTC-3' and reverse primer 5'-CGTGACTGGGAAAACCCTGG-3'; a *DpnI* digest was performed on the resulting mixture and then purified with a GeneJET PCR purification kit (Thermo Scientific). A double-stranded DNA fragment or gBlock (Integrated DNA Technologies) with a front overlapping segment (5'-CCAGGGTTTTCCCAGTCACG-3') and a rear overlapping segment (5'-CCACTTATTTTGGATCGTTTCGCTC-3') was introduced into the previously amplified pALS plasmid as per the instructions for the NEB Gibson Assembly Master Mix (New England Biolabs). Chemically competent DH10B cells were transformed with 5 µL of the mix following assembly. The rescued cells were plated on LB agar plates containing 25 µg/mL tetracycline and allowed to grow for 28 h at 37 °C. The vectors were purified with a QIAprep spin mini kit (Qiagen), and the tRNA sequences were verified.

Expression and Purification of Aminoacyl-tRNA Synthetases

BL21-AI cells containing a *pET-3NT8RS* or *pET-TyrRS* gene were grown overnight at 37 °C in 5 mL of noninducing medium supplemented with 100 µg/mL kanamycin.³⁷ A 50 mL culture of arabinose autoinduction medium (Table S1) supplemented with 100 µg/mL kanamycin and 0.02% lactose was then inoculated with a 1:100 dilution of the starter culture.³⁸ After 24 h at 37 °C, cells were pelleted and stored at -80 °C.³⁹

The best performing first-generation nitroTyrRS and wild-type TyrRS were purified using methods similar to those previously described.⁴⁰ Briefly, cells were resuspended in

approximately 10 mL of binding/wash buffer [20 mM sodium phosphate, 500 mM NaCl, and 20 mM imidazole (pH 7.4)], lysed once with a Microfluidics M-110P microfluidizer set at 18000 psi, and centrifuged at 20000 rcf for 25 min at 4 °C. The supernatant was filtered with an Acrodisc 32 mm syringe filter with a 0.45 Supor membrane before being applied to an ÄKTA Explorer FPLC system (GE Healthcare Life Sciences) fitted with a 1 mL HisTrap NiNTA column (GE). The column was washed with 20 mL of wash buffer and then eluted with a 0 to 100%, 30 mL linear gradient of elution buffer [20 mM sodium phosphate, 500 mM NaCl, and 500 mM imidazole (pH 7.4)]. Yields were approximately 15 mg/L of culture (Tyr-WT RS) and 180 mg/L of culture (nitroTyr-3NT8 RS). Fractions containing >95% pure nitroTyr-3NT8 and Tyr-WT RS as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis were pooled, dialyzed overnight into storage buffer [20 mM Tris, 50 mM NaCl, and 10 mM β -mercaptoethanol (pH 8.5)], concentrated to 20 mg/mL, frozen in liquid nitrogen, and stored at –80 °C. The second-generation nitroTyrRS-5B was expressed and purified as previously described.³⁰

Plasmid and Medium Conditions for the Measurement of Efficiency, Fidelity, and Orthogonality

All measurements of efficiency, fidelity, and orthogonality *in vivo* were performed in DH10B *E. coli* cells with a superfolder GFP reporter.³⁰ For these measurements, the cells contained some combination of a pALS-sfGFP-WT or pALS-sfGFP-150TAG plasmid, expressing the sfGFP gene and the tRNA, and a pBK plasmid expressing the aaRS. The kanamycin (Kn) resistant pBK plasmid encodes the aminoacyl-tRNA synthetase of interest under control of the constitutive *E. coli* GlnRS promoter and terminator.⁴¹ For efficiency measurements, cells containing both the pALS-sfGFP-150TAG and pBK plasmids were grown in media containing the ncAA of interest, with the level of GFP fluorescence reporting the efficiency of ncAA incorporation. Fidelity measurements were taken with cells containing the pALS-sfGFP-150TAG and pBK plasmids in media without the ncAA of interest. To assess the orthogonality of a particular tRNA sequence, cells containing the pALS-sfGFP-150TAG plasmid alone were grown in media without an ncAA.

Efficiency of NitroTyr Incorporation *In Vivo*

All protein expression was performed in DH10B cells containing either pALS-sfGFP or pALS-sfGFP-150TAG and a pBK vector to produce WT-sfGFP or nitroTyr-sfGFP. The arabinose autoinduction medium and protocol were used. Cultures were inoculated with 1:100 dilutions of noninducing cultures.³⁷ Expressions were performed in duplicate with 3 mL of autoinduction medium supplemented with 100 μ g/mL kanamycin and 25 μ g/mL tetracycline in 17 mm $\times$ 100 mm culture tubes being shaken at 250 rpm and 37 °C for 48 h. Error bars represent the standard deviation obtained from duplicate expressions. For the nitroTyr concentration-dependent sfGFP overexpression, nitroTyr was dissolved to a concentration of 10 mM in arabinose autoinduction medium, and 1 molar equiv of NaOH was added to help alleviate differences in cell growth due to media pH changes from the added amino acid. The nitroTyr stock was then serially diluted with medium to obtain the necessary concentrations of nitroTyr.

Fluorescence measurements of the cultures were collected 24 h after inoculation using a Synergy 2 Multi-Mode Reader (BioTEK). The emission and excitation wavelengths were set to 528/20 nm and 485/20 nm, respectively. Samples were prepared by placing 200 μ L of the cell suspension directly in Nunc MicroWell 96-well polypropylene plates (Sigma-Aldrich). The optical density of the cultures was determined at the same time by measuring the absorbance at 620 nm of a 1:10 dilution of the cells in Nunc MicroWell 96-well polystyrene clear flat bottom plates (Sigma-Aldrich). Cell fluorescence levels were normalized for the effects of OD and amino acids on fluorescence. To normalize for the effect of nitroTyr on sfGFP fluorescence, cells expressing WT sfGFP were grown in the presence of nitroTyr, normalized for OD, and the resulting fluorescence levels were normalized to each other. The resulting normalization factors were then applied to the remainder of the fluorescence data.

Efficiency of Incorporation of Various ncAAs with the C34/A37 Mutant tRNA

To determine the generality of the results for the C34/A37 mutant tRNA seen with nitroTyr, cells containing the pALS-sfGFP-150TAG plasmid with the C34/A37 mutant tRNA were cotransformed with pBK plasmids encoding genes for modified TyrRS enzymes selective for a variety of different ncAAs. The aaRS enzymes used were previously selected for incorporation of 4-benzoyl-L-phenylalanine (Bpa), 4-(trifluoromethyl)-L-phenylalanine (tfmF), 4-cyano-L-phenylalanine (pCNF), and acridon-2-ylalanine (Acd).^{42–44} Each pALS/pBK pair was grown in autoinduction medium, in the presence of their respective ncAA at 0.25mM (see Figure S2), for 48 h, with all other conditions and measurements as described above for nitroTyr. The ncAAs in this study were purchased from Peptech, Alfa Aesar, or Bachem or were synthesized as previously described.¹⁵

Preparation and Labeling of tRNA Transcripts for Aminoacylation

The tRNA substrates for aminoacylation by TyrRS and nitroTyrRS were prepared by *in vitro* transcription.⁴⁵ Because tRNA^{Tyr} and its variants begin with 5'-C1, which is unfavorable for transcription by T7 RNA polymerase, tRNAs were transcribed from DNA templates encoding a self-cleaving hammerhead ribozyme immediately upstream.⁴⁶ DNA templates were assembled from four synthetic oligonucleotides. Overlapping regions are underlined, and mutations were imposed at positions indicated in boldface (Table 1): (1) 5'-AATTCCTGCAGTAATACGACTCACTATAGGGAGACCGGCTGATGAGTC-3', (2) 5'-CCGGGACGGTACCGGGTACCGTTTCGTCCTCACGGACTCATCAGCCGGTCTCCC-3', (3) 5'-CCCGGTACCGTCCCGGCGGTAGTTCAGCCTGGTAGAACGGCGGACTGTAG-3', and (4) 5'-TGGTCCGGCGGGCCGGATTGTAACCAGCGACATGCGGATCTACAGTCCCGGTTCTACC-3'.

Assembly was facilitated by the Vent polymerase [New England Biolabs (NEB)]. A 50 μ L PCR was set up with 1 $\times$ Thermopol Buffer (NEB), each oligonucleotide at 2 μ M, each dNTP at 50 μ M, and 1 μ L of Vent polymerase (NEB). The reaction mixture was heated for 5 min at 94 °C and subjected to eight of the following PCR cycles: 94 °C for 1.5 min, 54 °C for 2 min, and 72 °C for 3 min. A fifth oligonucleotide was used, along with oligonucleotide 1, to amplify the assembled template: (5) 5'-[2'-OMe]U[2'-

OMe]GGTCCGGCGGGCCGG-3'. The 2'-*O*-methyl modifications inhibit runover transcription by T7 RNA polymerase.⁴⁷ A 400 μ L PCR was set up with 1 $\times$ Taq buffer (Thermo), 2.5 mM MgCl₂, each dNTP at 50 μ M, each oligonucleotide at 1 μ M (1 and 5), 4 μ L of the Vent reaction mixture, and 0.031 unit/ μ L Taq polymerase (Thermo). The reaction mixture was heated at 95 °C for 3 min and then subjected to 40 of the following cycles: 95 °C for 0.5 min, 60 °C for 0.5 min, and 72 °C for 0.17 min. The template was purified using a GeneJET Purification Kit (Thermo) and diluted into a 2 mL transcription reaction mixture containing 40 mM Tris-HCl (pH 8.0), 25mMMgCl₂, 2 μ M spermidine, 0.01% Triton X-100, 40 mM DTT, each NTP at 4 mM, 0.001 unit/ μ L inorganic pyrophosphatase (Roche), and 0.04 mg/mL T7 RNA polymerase^{172–173}, which was expressed and purified in the laboratory as described previously.⁴⁸ The reaction mixture was incubated at 37°C for 16–24 h.

To promote cleavage of the hammerhead ribozyme, the reaction mixture was diluted 5-fold into a buffer containing 40 mM Tris-HCl (pH 8.0), 25mMMgCl₂, 2 μ M spermidine, 0.01% Triton X-100, and 40 mM DTT and incubated at 60 °C for 4–7 h. Cleaved RNAs were concentrated to 0.5 mL and washed with TE6 buffer [10 mM BisTris-HCl and 1 mM EDTA (pH 6.0)] using a centrifugal filtration device [Amicon; 10 kDa molecular weight cutoff (MWCO)]. After proteins had been removed by extraction with 1 volume of a 25:24:1 phenol/chloroform/isoamyl alcohol mixture (pH 7.8) (Sigma), tRNA was purified away from uncleaved transcripts and free hammerhead ribozyme by electrophoresis through a 15 cm gel containing 15% polyacrylamide:bis(acrylamide) (29:1), 8 M urea, and 1 $\times$ TBE. tRNA was visualized by UV shadowing and excised from the gel. tRNA was extracted from the gel band with 10 volumes of TE6, shaken for 16–24 h at room temperature. Insoluble gel debris were removed by centrifugation at 5000 ref for 10 min. The supernatant was passed through a 0.45 μ m filter and washed with TE6 buffer using a centrifugal filtration device (Amicon; 10 kDa MWCO) such that urea was diluted to a concentration of <1 μ M.

The tRNA substrates for aminoacylation were radiolabeled at the 5'-phosphate of nucleotide A76 in accordance with established methods.⁴⁹ Labeling reactions (volume of 80 μ L) were performed in siliconized conical microcentrifuge tubes in a solution containing 20 mM glycine (pH 9.0), 4 mM MgCl₂, 2.5 mM pyrophosphate, 0.2 unit of pyrophosphatase (Roche), 2.5 μ M tRNA, ~50 μ Ci of [α -³²P]ATP, and ~5 μ M *E. coli* nucleotidyltransferase, purified in our laboratory as described previously.⁵⁰ Reactions were initiated by the addition of the nucleotidyltransferase and were allowed to proceed at 37 °C for 5 min. Prior to labeling, tRNA was denatured by being incubated for 10 min at 65 °C in ~50 μ L of water and refolded by being slowly cooled to room temperature over the course of 1 h in the presence of glycine and MgCl₂. Labeled tRNA was purified by extraction with 1 volume of a 25:24:1 (pH 7.8) phenol/chloroform/isoamyl alcohol mixture (Sigma) and purified from unincorporated ATP by electrophoresis through a 15 cm gel containing 15% polyacrylamide:bis(acrylamide) (29:1) and 1 $\times$ TBE. tRNA was visualized by phosphorimaging to facilitate excision from the gel. tRNA was extracted from the gel band with 2 volumes of TE6 buffer without shaking, in a 16–24 h incubation at ambient temperature.

Aminoacylation Reactions

Aminoacylation assays were conducted at 37 °C in siliconized conical tubes containing 20 μ L of 50mMHEPES-KOH (pH 7.5), 20mMNaCl, 10mMMgCl₂, 1 mM DTT, 2.5 mM MgATP, and various concentrations of amino acid, tRNA, and enzyme as appropriate. Methods used were similar to those employed in our laboratory for the study of other tRNA synthetases.^{51–53} Reactions were initiated by the addition of enzyme, which was diluted into the reaction mixture from a 10 $\times$ stock. tRNAs were refolded prior to aminoacylation as follows. Labeled and unlabeled tRNAs were diluted into TE6 buffer to yield a 5 $\times$ mixture, which was incubated at 65 °C for 10 min and then supplemented with 0.5 volume of 100 mM MgCl₂ that was prewarmed to 65 °C. The resulting tRNA/MgCl₂ mixture was then slowly cooled to room temperature over the course of 1 h. Time points were quenched by 1:3 dilution into a solution containing 200mMNaOAc (pH 5.2) and 0.2% SDS and subsequently digested for 15 min at room temperature with *Penicillium citrinum* P1 nuclease (Sigma) at a concentration of 0.0125 unit/ μ L. Free nucleotides were separated by thin layer chromatography across 10 cm PEI cellulose sheets (Sigma) with a solvent containing 1 M ammonium acetate and 5% acetic acid. Spots corresponding to AMP and aminoacyl-AMP were visualized by phosphorimaging and quantified using *imageJ*.⁵⁴

For short-term (<2 months) storage at –20 °C as 10 $\times$ stocks, enzymes were diluted into a buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM DTT, and 50% glycerol.

RESULTS

M. jannaschii Tyrosyl-tRNA Synthetase Efficiently Aminoacylates Unmodified Cognate tRNAs with Tyrosine

To establish an experimental system for measuring the detailed kinetic properties of TyrRS variants emerging from directed evolution, we first expressed and purified wild-type *M. jannaschii* TyrRS in *E. coli* and characterized its performance in the canonical two-step tyrosylation reaction using unmodified wild-type *M. jannaschii* tRNA^{Tyr} (wt-tRNA) produced by *in vitro* transcription with T7 RNA polymerase. tRNA was labeled at the 3'-internucleotide linkage using tRNA nucleotidyltransferase and subjected to refolding prior to use.⁴⁹ After tRNA refolding parameters had been evaluated, including the magnesium ion concentration and maximal temperature (see Materials and Methods for details), the maximal aminoacylation capacity of the transcript reached 67% in reactions performed with a molar excess of enzyme (Figure 1). The failure to achieve complete aminoacylation is common in *in vitro* aminoacylation reactions and may be due to a subpopulation of tRNAs that is kinetically trapped in a nonfunctional conformation.⁵⁵ In our hands, plateau aminoacylation at this level yields reliable kinetic parameters in other aaRS systems.⁵⁶

Steady-state kinetics were then performed to determine k_{cat} , K_m (tRNA), and K_m (Tyr) (Table 1). The k_{cat} of 1.0 s^{–1} and the K_m (tRNA) of 1.4 μ M indicate an efficiently performing cognate pair, suggesting that post-transcriptional modifications in the tRNA are unlikely to have strong effects on aminoacylation. Studies of bacterial tyrosylation also showed that modified nucleotides do not affect the kinetic efficiency in that system.⁵⁷ A K_m (Tyr) of 9.1

μM is very similar to the magnitude of the tyrosine equilibrium binding constant for bacterial TyrRS.⁵⁸

The k_{cat} that we measure is 20-fold higher than previously reported for aminoacylation of the same unmodified *M. jannaschii* tRNA^{Tyr} species (wt-tRNA), while $K_{\text{m}}(\text{tRNA})$ values are comparable.⁵⁹ The discrepancy is likely due to the use of just 11 μM tyrosine in the prior assays for $K_{\text{m}}(\text{tRNA})$ determination, a value almost identical to $K_{\text{m}}(\text{Tyr})$ and thus affording only partial enzyme saturation (Table 1). The prior study also used 10 mM ATP,⁵⁹ a level that we have found to be inhibitory (data not shown; high concentrations of ATP may inhibit by competing for binding with the 3'-terminal A76 of tRNA).⁶⁰ These findings emphasize the value of monitoring aminoacylation through the use of ³²P-labeled tRNA. We used 48 μM tyrosine in the $K_{\text{m}}(\text{tRNA})$ determinations, 5-fold above $K_{\text{m}}(\text{Tyr})$ (Table 1). The ATP concentration of 2.5 mM was also confirmed to be saturating (data not shown). These measurements show that *M. jannaschii* TyrRS is an efficient aaRS and does not exhibit a slow k_{cat} as previously reported.

Correlating *In Vivo* Performance with Kinetic Measurements

We next compared the *in vivo* expression yields of nitroTyr-sfGFP by first- and second-generation nitroTyrRS enzymes, as a function of the concentration of nitroTyr included in the autoinduction expression medium (Figure 2). The first- and second-generation enzymes used in these experiments were the best performers among the isolates from those rounds of directed evolution.^{29,30} The crystal structure of the best-performing second-generation enzyme is also available in apo and nitroTyr-bound states.³⁰ As previously demonstrated, the second-generation nitroTyrRS is substantially more efficient than the first-generation enzyme at all nitroTyr concentrations tested. This enzyme exhibits saturation at a nitroTyr concentration of approximately 0.2 mM, while the first-generation variant produces a weakly increasing level of nitroTyr-sfGFP up to the highest nitroTyr concentration tested (Figure 2). For reference, native sfGFP without an amber stop codon expresses at levels in autoinduction medium 5-fold higher than that of the second-generation nitroTyr aaRS under identical conditions supplemented with 1 mM nitroTyr.³⁰

The determination of sfGFP yield *in vivo* at differing nitroTyr concentrations allows the possibility of a quantitative correlation with the catalytic performance of the enzymes *in vitro*. To examine this, we expressed and purified both first- and second-generation nitroTyrRS variants from *E. coli* and characterized aminoacylation by steady-state kinetics, as described above. The first-generation enzyme exhibits very weak nitrotyrosylation of wt-tRNA, with plateau levels of aminoacylated product much too low for determination of kinetic parameters (Figure 1). In contrast, the second-generation nitroTyrRS exhibits robust catalytic performance, with its $K_{\text{m}}(\text{tRNA})$ being identical to that of wild-type TyrRS and its k_{cat} diminished by only ~4-fold (Table 1). Comparative measurements under identical single-turnover conditions suggest that the first-generation enzyme performs approximately 1000-fold less efficiently, although precise estimates are difficult because we do not observe saturation for nitroTyr in that case (data not shown). These findings show that the much higher levels of nitroTyr incorporation by the second-generation enzyme are substantially based on direct catalytic improvement of the evolved nitro-TyrRS.

Further measurements with the second-generation enzyme showed that a $K_m(\text{nitroTyr})$ of 10mM is ~1000-fold higher than the $K_m(\text{Tyr})$ in WT-TyrRS (Table 1). However, the X-ray structure of the enzyme:nitroTyr complex offers no indication of weak binding, revealing instead a specific set of interactions with the nitroTyr substrate, including stacking interactions with the ring and several hydrogen bonds with the exocyclic nitro group.³⁰ Consistent with the high measured K_m value, nitroTyr was not observed in electron density maps when included at a concentration of 2 mM in crystallization drops but appeared only when the crystals were later soaked in solutions containing this ligand at a concentration of 100 mM.³⁰ A possible explanation for this apparent discrepancy is that the crystal structure was determined without tRNA, while $K_m(\text{nitroTyr})$ reflects the performance in its presence (see Discussion).

Next, we repeated kinetic measurements for tyrosylation by WT-TyrRS and nitrotyrosylation by the second-generation enzyme, this time using an *M. jannaschii* tRNA^{Tyr} transcript corresponding to the “orthogonalized” species present in the cells during both the directed evolution experiments and the measurements of sfGFP production.³⁰ This permits a more precise correlation of *in vivo* performance with *in vitro* aminoacylation efficiency. The orthogonalized tRNA (orthog-tRNA) was isolated using genetic selections when the *M. jannaschii* TyrRS:tRNA^{Tyr} system was first developed,¹² and to the best of our knowledge, this particular species has been used in all subsequent directed evolution experiments with the *M. jannaschii* TyrRS scaffold. Orthog-tRNA possesses six nucleotide substitutions, comprising four replacements in the D and variable loop portions of the tertiary core (C17A/U17aG/U20G/U47G), and two replacements in the anticodon loop at the wobble position (G34C, as required to create the amber suppressor) and position 37 (G37A, located immediately 3' to the anticodon sequence) (Figure 3).

Orthog-tRNA elicits responses similar to those of a substrate for TyrRS and nitroTyrRS. k_{cat} is unchanged for nitroTyrRS and decreased ~2-fold for wild-type TyrRS, but $K_m(\text{tRNA})$ is very substantially elevated for both enzymes (by 16-fold for TyrRS and 27-fold for nitroTyrRS) (Table 1). Thus, orthog-tRNA exhibits reduced complementarity for both TyrRS and nitro-TyrRS compared to that of wild-type tRNA^{Tyr}. Remarkably, both enzymes also show quantitatively similar decreases in $K_m(\text{amino acid})$ (13-fold for wild-type TyrRS with tyrosine and 17-fold for nitroTyrRS with nitroTyr) (Table 1), suggesting substantially improved complementarity with this substrate. Because the six substitutions in orthog-tRNA are located 15–40 Å from the amino acid binding pocket on each protein, these observations strongly suggest that long-distance intermolecular communication between the tRNA and amino acid binding sites is a key mechanistic feature of both enzymes. Because tRNA K_m values are so high with orthog-tRNA, saturation for tRNA could not be maintained in the experiments measuring $K_m(\text{amino acid})$. Thus, amino acid K_m values should be regarded as apparent values in these cases (Table 1).

Because nitroTyr-sfGFP synthesis *in vivo* depends on the function of nitroTyrRS, the nitroTyr concentration dependence of nitroTyr-sfGFP formation could bear some relation to the apparent K_m value for nitroTyr exhibited by the evolved enzyme (Figure 2). In fact, the nitroTyr concentration giving half-maximal nitroTyr-sfGFP production is slightly less than 0.1 mM, ~6-fold below the relevant apparent K_m of 0.56 mM (Table 1). The quantitative

relationship between these parameters will, of course, be influenced by differing ATP, tRNA, and ncAA concentrations in the two settings, and probably by other factors, as well (see Discussion).

Dissecting the Orthogonalized *M. jannaschii* tRNA^{Tyr}

The *M. jannaschii* tRNA^{Tyr} identity is specified by the C1-G72 base pair in the acceptor stem, the single-stranded A73 nucleotide located adjacent to the 3'-CCA sequence, and the full anticodon triplet G34-U35-A36 (Figure 3).⁵⁹ Introduction of these six identity nucleotides into a noncognate tRNA framework conferred full tyrosylation capacity,⁵⁹ showing that no other nucleotides are essential. Among these six nucleotides in wild-type tRNA^{Tyr}, the only one mutated in orthog-tRNA is G34, which must be substituted with C34 to create the amber suppressor. The necessity of this mutation at an identity position might be expected to diminish the quality of the *M. jannaschii* TyrRS:tRNA^{Tyr} complex as an orthogonal pair for ncAA incorporation at amber codons. However, the kinetic measurements described above show that, while $K_m(\text{tRNA})$ is significantly elevated for the G34C tRNA with both TyrRS and nitroTyrRS, $K_m(\text{Tyr})$ and $K_m(\text{nitroTyr})$ are lowered by nearly the same magnitude in the respective enzymes (Table 1). Thus, the direct interaction of TyrRS with the tRNA^{Tyr} anticodon, by which the G34 identity is expressed (Figure 3), may not be entirely detrimental (see Discussion).

The crystal structure of *M. jannaschii* TyrRS bound to tRNA^{Tyr} and L-tyrosine shows that the core region of the tRNA does not interact with the enzyme, suggesting that the four orthog-tRNA mutations in the D and variable loops may not provide positive determinants for TyrRS aminoacylation. However, the base of G37 stacks between G38 and anticodon nucleotide U36 and makes steric contacts with a nearby protein element (Figure 3).⁶¹ While G37 was not identified as an identity nucleotide, these interactions and the proximity of the G34C and G37A substitutions in orthog-tRNA nonetheless suggest that the nucleotide at position 37 could influence tyrosylation and/or nitrotyrosylation efficiencies. To evaluate the importance of the replaced nucleotides in orthog-tRNA, we generated the G34C and G34C/G37A mutants in wt-tRNA and examined their abilities to efficiently synthesize nitroTyr-sfGFP *in vivo*. For G34C, the yields of nitroTyr-sfGFP are lower than for the fully orthogonalized suppressor at all nitroTyr concentrations tested (Figure 4A). However, G34C/G37A is significantly more efficient than orthog-tRNA, particularly at low concentrations of nitroTyr. Hence, for nitroTyr incorporation by the second-generation enzyme, the four additional mutations in the D and variable loops are deleterious to nitroTyr-sfGFP synthesis *in vivo*.

We next examined how G34C and G34C/G37A tRNA^{Tyr} compare to wt-tRNA and orthog-tRNA as substrates for *in vitro* aminoacylation. As expected given the role of G34 as an identity determinant, G34C tRNA^{Tyr} exhibits a 20-fold reduced k_{cat} with TyrRS, while $K_m(\text{tRNA})$ is elevated by 2-fold (Table 1). Remarkably, however, $K_m(\text{Tyr})$ is sharply reduced by 30-fold. This large decrease in the amino acid K_m mirrors similar findings with orthog-tRNA and pinpoints the wobble base interactions as a key trigger for long-distance intramolecular communication to the amino acid binding pocket some 40 Å distant. Further, the small effect on $K_m(\text{tRNA})$ with this singly substituted species suggests that the altered

wobble base contacts are not primarily responsible for the substantially weakened orthog-tRNA complementarity with TyrRS (Table 1). Because the G34C/G37A double mutant exhibits kinetic parameters with TyrRS that are very similar to those with G34C alone, it appears that the four substitutions in the D and variable loops are responsible for most of the weakened tRNA complementarity. However, k_{cat} for aminoacylation of orthog-tRNA by TyrRS is substantially higher than for G34C or G34C/G37A, so the four tRNA core region substitutions in orthog-tRNA do rescue this kinetic parameter (Table 1).

Although nitroTyrRS differs from TyrRS at just five residues in the amino acid substrate binding pocket, it does not respond similarly to substitutions at the anticodon loop wobble position. Most notably, $K_m(\text{nitroTyr})$ is decreased by only ~4-fold with G34C tRNA, substantially less than the 17-fold decrease in $K_m(\text{nitroTyr})$ observed with orthog-tRNA, and well below the 30-fold reduction in $K_m(\text{Tyr})$ by TyrRS with G34C tRNA (Table 1). Another very significant difference between TyrRS and nitroTyrRS is that $K_m(\text{tRNA})$ for the G34C tRNA with nitroTyrRS is greatly elevated: saturation could not be observed even at tRNA concentrations of >50 μM . This contrasts with the modest 2-fold increase in $K_m(\text{tRNA})$ for the G34C species with TyrRS. These distinctions in the enzymes' response to a single nucleotide substitution in the anticodon highlight the very strong interplay between amino acid and tRNA binding in this system, a characteristic that should influence the design of further directed evolution studies (see Discussion).

The kinetic measurements do not reveal large differences in steady-state nitrotyrosylation parameters between G34C and G34C/G37A tRNAs, although stronger effects may be present that are not revealed because of the technical limitation associated with weak tRNA binding. However, even without a precise determination, it is clear that k_{cat} for G34C/G37A is larger than for G34C, while $K_m(\text{nitroTyr})$ is ~2-fold lower. These differences may explain the 5-fold lower ncAA concentration needed to reach half-maximal nitroTyr-GFP expression for G34C/G37A compared with that for G34C *in vivo*. The concentration of nitroTyr needed to reach half-maximal expression of nitroTyr-GFP is 3-fold lower for G34C/G37A than for the orthog-tRNA, although the basis for improved nitroTyr-sfGFP synthesis is not apparent from these kinetic measurements (see Figure 4A and Discussion).

Absolute Fidelity and Orthogonality of tRNA^{Tyr} Suppressors *in Vivo*

To assess whether the variations among orthog-tRNA, G34C, and G34C/G37A affect the ability of nitroTyrRS to discriminate nitroTyr from other amino acids, we measured the sfGFP yields *in vivo* for each of these suppressors, both with and without the addition of nitroTyr to the culture medium. Because nitroTyr is not present in *E. coli*, any observed fluorescence from sfGFP must arise from aminoacylation of other amino acids by nitroTyrRS. The data reveal that all three strains permit a low level of sfGFP synthesis in the absence of nitroTyr, but the G34C and G34C/G37A suppressors are only 25% less selective for amino acids than orthog-tRNA (Figure 4B).

Next, we conducted similar experiments to measure yields of sfGFP with each of the three tRNAs, in the presence and absence of nitroTyrRS. All of these experiments were conducted in the absence of nitroTyr. Synthesis of sfGFP in the absence of nitroTyrRS indicates that read through of the amber codon occurs by misacylation of a tRNA^{Tyr} suppressor with a

canonical amino acid, by another aaRS in the cell. This measures the extent to which the engineered nitroTyrRS:tRNA^{Tyr} pair is orthogonal to the endogenous aaRS:tRNA systems. Here we find that G34C tRNA^{Tyr} exhibits a 4-fold increase in the level of misacylation compared to that of orthog-tRNA in the absence of the aaRS, but only a 2-fold increase when the aaRS is present (Figure 4C). However, both G34C/G37A and orthog-tRNA fully alleviate this effect, demonstrating that the four nucleotide substitutions in the D and variable loops of orthog-tRNA are not required to maintain orthogonality.

Effect of Orthogonalized *M. jannaschii* tRNA^{Tyr} on Other ncAA tRNA/RS Pairs

To assess whether the superior performance of G34C/G37A tRNA for incorporation of nitroTyr can also be applied to the incorporation of other ncAAs, we measured ncAA-sfGFP synthesis in the presence of four other evolved *M. jannaschii* TyrRS enzymes. These aaRS enzymes were selected for 4-benzoyl-L-phenylalanine (Bpa), 4-(trifluoromethyl)-L-phenylalanine (tfmF), 4-cyano-L-phenylalanine (pCNF), and acridon-2-ylalanine (Acd).^{42–44} To evaluate the pCNF-RS, we used 4-azido-L-phenylalanine because it is an extensively used cross-linker. In each case, we compared the performance of G34C/G37A with orthog-tRNA, in the presence and absence of 0.25mMncAA in the culture medium (Figure 5). This was chosen as the lowest concentration that can maximize incorporation of nitroTyr with G34C/G37A tRNA (Figure 4A). We find that evolved aaRSs specific for Bpa, tfmF, and pCNF each incorporate the ncAA more efficiently with G34C/G37A, with a particularly large improvement (340%) observed for Bpa (Figure 5). In contrast, the evolved AcdRS generated more than twice as much ncAA-sfGFP when paired with orthog-tRNA rather than G34C/G37A. Therefore, whether and the extent to which the G34C/G37A tRNA performs better than orthog-tRNA depend on which ncAA is incorporated. This provides yet another demonstration of the importance of intramolecular signaling between the amino acid and tRNA binding sites of evolved TyrRS enzymes. Although the effects are weak, experiments in the absence of added ncAA suggest that the fidelity of amino acid incorporation is slightly compromised when G34C/G37A tRNA is substituted for orthog-tRNA, for all five ncAAs tested (Figure 5).

DISCUSSION

Our findings show that the efficiency of ncAA incorporation by evolved *M. jannaschii* TyrRS enzymes depends on nucleotide determinants in the cognate tRNA^{Tyr} suppressor. Four of five tRNA nucleotides previously identified by directed evolution are deleterious to the efficiency of ncAA-sfGFP production in *E. coli*, while the G37A substitution alone, when paired with the wobble mutation G34C, substantially improves incorporation for four of the five tested ncAAs (Figure 5). We confirm the earlier conclusion that the single mutant G34C tRNA^{Tyr} suppressor is not orthogonal in *E. coli* (Figure 4C).¹² However, our findings additionally demonstrate that tRNAs selected from large libraries for purposes of improving orthogonality may possess other nucleotide substitutions that are deleterious for efficient protein synthesis. Clearly, negative tRNA determinants that block noncognate aaRS interactions *in vivo* might also affect the catalytic efficiency of the desired cognate reaction. As we have shown here, systematic testing of mutations found in evolved tRNAs can distinguish which new substitutions are useful and which are not. We expect that the new

G34C/G37A *M. jannaschii* tRNA^{Tyr} should improve ncAA incorporation for many, although not all, of the recombinant proteins that have been so far generated with orthog-tRNA in this system.

Steady-state measurements of aminoacylation kinetics are useful in characterizing evolved, orthogonal aaRS:tRNA pairs, as previously demonstrated for the PylRS system.^{17,28} Here, these measurements clearly show that a major improvement in the second-generation nitroTyrRS derives from improved aminoacylation efficiency (Figure 1). Kinetics also helps to pinpoint differences in catalytic function between wild-type and orthogonal components, to identify where further improvements might be made. The most remarkable finding is that, while K_m for orthog-tRNA is elevated for both TyrRS and nitroTyrRS, K_m for amino acid is sharply decreased by ~15-fold in both enzymes (Table 1). This demonstrates sizable structural coupling between amino acid and tRNA recognition in *M. jannaschii* TyrRS, of a magnitude substantially greater than that previously characterized in the well-studied bacterial glutamyl-tRNA synthetase (GlnRS) system.⁶² The unexpected improvement in amino acid binding engendered by tRNA mutation suggests that the enzyme-tRNA interface has evolved to maintain weak amino acid binding by the protein component of the complex.

This very weak amino acid binding could be an optimization reached on the basis of intracellular concentrations of all amino acids, tRNAs, and aaRS in *M. jannaschii*, as required to maintain the fidelity of protein synthesis, and perhaps influenced by the extreme environment that is inhabited by this hyperthermophilic anaerobe. The interrelatedness of tRNA and amino acid binding also has implications for further design of TyrRS and tRNA^{Tyr} to improve performance *in vivo*. It is important to note that no selections have yet been performed using the optimized G34C/G37A tRNA^{Tyr}; such experiments could yield novel and better-performing nitroTyrRS variants, and as mentioned, this new suppressor should also be of broad utility for other ncAAs. We also note that the major catalytic detriment in the system remains the very high K_m for tRNA. Thus, selections incorporating enzyme libraries that target the tRNA interface in regions spanning the anticodon to the active site could be fruitful, while maintaining the already evolved amino acid binding sites. This may build on the work of Guo and colleagues, who were able to improve incorporation of some ncAAs by *M. jannaschii* TyrRS by manipulating protein residues at the anticodon interface of orthog-tRNA.^{13,27} It might also be possible to identify constellations of tRNA core region nucleotides that, rather than being detrimental as in orthog-tRNA, might instead improve tRNA binding through indirect modulation of the sugar-phosphate backbone conformation at the inner elbow portion of the tRNA L shape. Rationally engineered variants of *E. coli* GlnRS that incorporate noncognate glutamate manifested improved function when conserved tRNA^{Glu} core region nucleotides were substituted into the GlnRS:tRNA ribonucleo-protein.⁶³

Our data also illustrate how measurement of aminoacylation kinetics is necessary but certainly not sufficient for a thorough understanding of how engineered orthogonal aaRS:tRNA complexes function in the cell. $K_m(\text{nitroTyr})$ is not a quantitative proxy for the efficiency of nitroTyr uptake and incorporation *in vivo* (Figure 2 and Table 1); the discrepancy between this parameter and the concentration of nitroTyr giving half-saturation may reflect other factors such as limitations in cellular uptake of nitroTyr or downstream

aspects of protein synthesis. Characterization of EF-Tu binding kinetics and ribosomal performance may thus also be helpful in inspiring further improvement of TyrRS-based orthogonal systems. Ultimately, sophisticated applications in synthetic biology demand that the catalytic parameters of evolved aaRS should fully match those of endogenous aaRS in the host organism with respect to k_{cat} , $K_{\text{m}}(\text{tRNA})$, and $K_{\text{m}}(\text{amino acid})$. Thus, the 100-fold lower k_{cat} of *Methanosarcina barkeri* PylRS compared to that of TyrRS,²⁸ while not deleterious in the host cell due to the very small number of pyrrolysine codons, may be a disadvantage if the PylRS system is ultimately to provide a scaffold for extensive incorporation of ncAAs in genomically recoded microorganisms with alternate genetic codes.¹¹ In contrast, the perceived disadvantage of TyrRS in directly recognizing the anticodon, while still posing a challenge as shown by the high $K_{\text{m}}(\text{tRNA})$, is nonetheless mitigated by our findings that $K_{\text{m}}(\text{amino acid})$ is improved by tRNA mutation while k_{cat} is only modestly decreased (Table 1). Efforts in the synthetic biology of protein translation will doubtless also be promoted by the development of additional efficient aaRS scaffolds for ncAA incorporation.^{13,24}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Thin layer chromatograph (left) depicting results from an aminoacylation time course in which wild-type *M. jannaschii* TyrRS attaches tyrosine (Y) to a wild-type *M. jannaschii* tRNA^{Tyr} transcript. The lane at the right shows plateau aminoacylation levels after 20 min, performed under conditions. Thin layer chromatograph (right) depicting results from an aminoacylation time course in which the second-generation nitroTyrRS attaches nitroTyr to orthog-tRNA (see the text for nomenclature). The lane at the right shows plateau aminoacylation levels after 20 min. Plateau levels are as follows: wild-type TyrRS:tRNA^{Tyr}, 66%; wild-type TyrRS:orthog-tRNA, 50% (data not shown); second-generation nitroTyrRS:tRNA^{Tyr}, 62% (data not shown); second-generation nitroTyrRS:tRNA-orthog, 45%. (B) Aminoacylation time courses for the first-generation enzyme at the aaRS and nitroTyr concentrations noted. The tRNA concentration was 5 nM. (C) Plot of the apparent rate constant k_{obs} for reactions of the first- and second-generation nitroTyrRS enzymes as a function of nitroTyr concentration. These experiments were conducted using wild-type tRNA^{Tyr}. Note that the concentration of the first-generation enzyme is 200-fold greater than that of the second-generation enzyme. Only one of two replicates is shown for each enzyme.

Figure 2.

In vivo fluorescent measurement of nitroTyr-150-sfGFP expression as a function of nitroTyr concentration in the medium using the first-generation (red) and second-generation (blue) nitroTyrRS enzymes. Values shown represent the mean of two experiments, and the error bars shown indicate the standard deviation of each measurement.

Figure 3.

Cloverleaf depiction (left) of the sequence of wild-type *M. jannaschii* tRNA^{Tyr}. The six positions at which the tRNA sequence was altered to generate orthog-tRNA are circled, with the replaced nucleotide indicated. Crystal structure (middle) of *M. jannaschii* TyrRS bound to tRNA^{Tyr} and L-tyrosine (Protein Data Bank entry 1J1U). The two subunits of the dimeric enzyme are colored blue and purple. The tRNA anticodon is boxed at the bottom. Positions corresponding to substitutions in tRNA-orthog are indicated in one of the tRNAs. Detail (right) from the crystal structure of the TyrRS:tRNA^{Tyr}:L-tyrosine complex, showing hydrogen bonding and other contacts between TyrRS and the tRNA anticodon loop. Hydrogen bonds are depicted as dashed lines.

Figure 4.

(A) *In vivo* fluorescent measurement of nitroTyr-150-sfGFP expression using the second-generation nitroTyrRS with tRNA-orthog (blue), G34C tRNA (red), and G34C/G37A tRNA (green), as a function of nitroTyr concentration. (B) *In vivo* fluorescent measurement of nitroTyr-150-sfGFP expression for the respective suppressor tRNAs in the presence and absence of nitroTyr, in the presence of the second-generation nitroTyrRS. (C) *In vivo* fluorescent measurement of nitroTyr-150-sfGFP expression for the respective suppressor tRNAs in the presence and absence of the second-generation nitroTyrRS. Values shown represent the mean of two experiments, and the error bars shown indicate the standard deviation of each measurement.

Figure 5.

In vivo fluorescent measurement of nitroTyr-150-sfGFP expression, using five ncAAs together with the five distinct aaRS obtained from prior directed evolution experiments. Each enzyme/ncAA pair is tested with orthog-tRNA and G34C/G37A tRNA. Each experiment was conducted in the presence and absence of the relevant ncAA to assess ncAA-GFP efficiency and fidelity. See Figure S2 for ncAA structures. Values shown represent the mean of two experiments, and the error bars shown indicate the standard deviation of each measurement.

Table 1

tRNA	TyrRS: Tyr ^d				nitroTyrRS: nitroTyr			
	k_{cat} (s ⁻¹)	$K_{\text{MLR}}(\mu\text{M})$	$k_{\text{cat}}/K_{\text{MLR}}(\text{s}^{-1}\mu\text{M}^{-1})$	$K_{\text{M,aa}}(\mu\text{M})$	k_{cat} (s ⁻¹)	$K_{\text{MLR}}(\mu\text{M})$	$k_{\text{cat}}/K_{\text{MLR}}(\text{s}^{-1}\mu\text{M}^{-1})$	$K_{\text{M,aa}}(\text{mM})$
WT	1.0 ± 0.2	1.4 ± 0.1	0.7 ± 0.05	9.1 ± 0.5	0.26 ± 0.03	1.14 ± 0.13	0.23 ± 0.004	9.6 ± 6.1
G34C	0.05 ± 0.01	3.0 ± 1.3	0.02 ± 0.005	0.31 ± 0.01	<0.08 ^b	>16	0.007 ± 0.001	2.3 ± 0.2
G34C/G37A	0.13 ± 0.04	4.8 ± 1.6	0.018 ± 0.009	0.33 ± 0.18	<0.2 ^b	>16	0.01 ± 0.001	1.4 ± 0.2
orthog	0.52 ± 0.04	21.2 ± 2.0	0.02 ± 0.001	0.72 ± 0.04	0.31 ± 0.03	30.8 ± 3.6	0.01 ± 0.0003	0.56 ± 0.02

^a Sample Michaelis–Menten plots for each reaction are provided in Figure S1.

^b Only the upper bound for k_{cat} is indicated, because tRNA could not be saturated (see the text).