Published in final edited form as:
Biochemistry. 2011 February 15; 50(6): 1101–1109. doi:10.1021/bi101360a.

Fidelity escape by the unnatural amino acid β-hydroxynorvaline: an efficient substrate for *Escherichia coli* threonyl-tRNA synthetase with toxic effects on growth†

Anand Minajigi[‡], **Bin Deng**[‖], and **Christopher S. Francklyn, Ph.D.**[‡,§,*]

[‡]Cell and Molecular Biology Program, College of Medicine, Health Sciences Complex, 89 Beaumont Ave, University of Vermont, Burlington, VT 05405-0068

[‖]Department of Biology, University of Vermont, Burlington, VT 05405-0068

[§]Department of Biochemistry, College of Medicine, Health Sciences Complex, 89 Beaumont Ave, University of Vermont, Burlington, Vermont 05405

Abstract

In all living systems, the fidelity of translation is maintained in part by the editing mechanisms of aminoacyl-tRNA synthetases (ARSs). Some non-proteogenic amino acids, including β-hydroxynorvaline (HNV) are nevertheless efficiently aminoacylated and become incorporated into proteins. To investigate the basis of HNV's ability to function in protein synthesis, the utilization of HNV by *E. coli* threonyl-tRNA synthetase (ThrRS) was investigated through both *in vitro* functional experiments and bacterial growth studies. The measured specificity constant (k_{cat}/K_M) for HNV was found to be only 20–30 fold less than that of cognate threonine. The rate of aminoacyl transfer (10.4 s^{-1}) was 10-fold higher than the multiple turnover k_{cat} value (1 s^{-1}), indicating that, as for cognate threonine, amino acid activation is likely to be the rate-limiting step. Like non-cognate serine, HNV enhances the ATPase function of the synthetic site, at a rate not increased by non-aminoacylatable (3′-dA76) tRNA. ThrRS also failed to exhibit post-transfer editing activity against HNV. In growing bacteria, the addition of HNV dramatically suppressed growth rates, which indicates either negative phenotypic consequences associated with its incorporation into protein, or inhibition of an unidentified metabolic reaction. The inability of wild ThrRS to prevent utilization of HNV as a substrate illustrates that, for at least one ARS, the naturally occurring enzyme lacks the capability to effectively discriminate against non-proteogenic amino acids that are not encountered under normal physiological conditions. Other examples of 'fidelity escape' in the ARSs may serve as useful starting points in the design of ARSs with specificity for unnatural amino acids.

Aminoacyl-tRNA synthetases establish the fidelity of the genetic code in a two-step aminoacylation reaction, covalently linking specific amino acids to their corresponding tRNAs. In the first step, amino acid is condensed with ATP to form the aminoacyl adenylate (adenylation), while in the second step, the amino acid moiety of adenylate is transferred to the A76 of tRNA (aminoacyl transfer), along with the release of pyrophosphate (PPi) and AMP, respectively. ARSs have been divided into two classes, Class I and Class II, based on

†This work was supported by a grant from the National Institutes of Health (GM-54899 to C.S.F.) and by a grant from the National Institutes of Health, INBRE Program of the National Center for Research Resources (P20 RR16462 to J.V.H.)

*Address correspondence to: Christopher S. Francklyn, Ph.D., Department of Biochemistry, University of Vermont, Health Sciences Complex, 89 Beaumont Avenue, Burlington, Vermont 05405. TEL: 802-656-8450; FAX: 802-862-8229; Christopher.Francklyn@uvm.edu.

characteristic signature motifs, unique catalytic folds, and the regiochemistry of tRNA aminoacylation. Each ARS is specific for its corresponding amino acid and tRNA. Defects in the specificity of the ARS reaction can have significant physiological effects, including neurodegenerative diseases and other complex phenotypes (1). ARSs can also introduce errors into the translation process, which has a frequency of 1 mistake per $10^3 – 10^4$ amino acids incorporated into proteins (2,3).

Near-cognate amino acids that differ by a single methyl group from standard amino acids pose a particularly difficult discrimination problem (2,3). The limited amount of additional binding energy associated with a single methyl group imposes a significant requirement for specific error correction mechanisms (4). Among the first detailed explanation of ARS editing was the “double sieve” model proposed by Fersht (5). In this model, the first “coarse sieve” activates cognate as well as smaller and isosteric amino acids, while the second “fine sieve”, serves to edit only smaller or isosteric amino acids. During aminoacylation, amino acids can be edited either prior to aminoacyl transfer, which is referred as “pre-transfer editing”, or after transfer to the tRNA, known as “post-transfer editing”. Pre-transfer editing involves the hydrolysis of near-cognate adenylate, either in the enzyme active site, or by its release into solvent with subsequent hydrolysis (6,7). For some ARS, this process can be modulated by tRNAs (8,9). Post-transfer editing is characterized by a deacylation reaction that occurs in a separate active site localized to its own discrete editing domain. For Class I IleRS, ValRS, and LeuRS, this separate domain is referred to as CPI, and it is structurally distinct from the canonical Rossmann fold domain housing the aminoacylation active site (10). MetRS, which edits exclusively through a pre-transfer mechanism, lacks such a CPI domain (11). The class II ARSs, which possess robust post-transfer editing functions, including ProRS, ThrRS, AlaRS, and PheRS, have separate editing domains that are structurally unrelated to the class I CPI domain (12–16). A final mechanism is “resampling”, which involves the dissociation of misacylated tRNA from the synthetic site, followed by its rebinding and hydrolysis in the editing site (17). A critical part of this mechanism is the kinetic partitioning between rebinding to the editing site and capture by EF-Tu, which would channel the misacylated tRNA into protein synthesis.

Recently, there has been significant interest in the modification and engineering of the ARS-catalyzed aminoacylation reaction with the goal of enhancing the incorporation of unnatural amino acids (UAAs) into proteins (18). ARSs with relaxed or modified amino acid specificity have been developed, allowing non-standard amino acids to be introduced into bacterial proteins (19,20). In addition to expanding the range of unnatural amino acids that can be incorporated by ARSs engineering, recent work indicates that a number of the unmodified ARSs (especially those specific for non-polar amino acids) can tolerate a relatively broad range of unnatural amino acids (21). One conclusion from these studies is that the efficiency of incorporation of unnatural amino acids into proteins is roughly proportional to their propensity to serve as substrates for aminoacylation (22). Even though a number of unnatural amino acids have been identified that are readily incorporated into proteins, little work has been reported on how these compounds are treated by the ARS during the individual steps of the overall reaction, including proofreading steps. One non-standard α-amino acid that is not present in standard bacterial or eukaryotic cell physiology and is incorporated into peptides in cell free extracts is β-hydroxynorvaline (HNV), a near-cognate analog of threonine (21,23) Therefore, to gain a detailed understanding of how ThrRS deals with a near-cognate unnatural amino acid like HNV, steady state kinetics, pre-steady state analysis, and *in vivo* bacterial growth experiments were performed. Consistent with the ease with which this threonine analog is accepted by the protein synthesis machinery, we observed that ThrRS possesses essentially no ability to discriminate against HNV, in any of the individual steps of the aminoacylation reaction.

EXPERIMENTAL PROCEDURES

Expression and purification of protein and tRNA

The expression and purification of His-tagged ThrRS and CCA adding enzymes was described previously (24). Purification of both proteins involved the use of Ni-NTA affinity column chromatography. For both proteins, the final fractions were pooled, dialyzed against Buffer A (10 mM Tris pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 3 mM β-mercaptoethanol), and then concentrated under low speed centrifugation using Millipore concentrators, prior to storage in 50% glycerol at −20 °C. The active site concentrations of ThrRS preparations (25) were determined before performing the kinetic experiments. The fully modified $tRNA^{Thr}$ was over-expressed in bacteria and purified by phenol extraction, followed by size fractionation on urea-PAGE (24). The band corresponding to $tRNA^{Thr}$ was excised from the gel, electroeluted, and then the eluate was ethanol precipitated. The concentrations of $tRNA^{Thr}$ in the preparations were determined from plateau aminoacylation assays (25).

Amino acid competition experiments

Aminoacylation reactions were performed at 37 °C and pH 8 in Buffer A, essentially as described (25). Reactions included 0.25 μM wild type ThrRS, 10 μM $tRNA^{Thr}$, 2.5 mM ATP, 0.2 U/mL pyrophosphatase (PPase), (100 μM [^{14}C] threonine, in the absence or presence of different concentrations of HNV (0.2, 2, and 10 mM). At different time points, reaction aliquots were taken, quenched on trichloroacetic acid (TCA) soaked discs, and then processed according to standard aminoacylation procedures (25). The discs were dried and counted in a scintillation counter. The formation of product was plotted on the y-axis, while time was plotted on the x-axis for analysis.

Steady state aminoacylation

The preparation of $tRNA^{Thr}$ labeled with [^{32}P] at A76 was accomplished as described in (26); this material was then used as the radioactive substrate in the aminoacylation experiments. The reactions were carried at 37 °C in Buffer A along with 2.5 mM ATP, 6 μM $tRNA^{Thr}$, 0.2 U/mL PPase, trace amount of [^{32}P] tRNA, and 10–25 nM wild type ThrRS enzyme. The reactions were quenched and digested with S1 nuclease for at least 1 hr. The HNV-[^{32}P] A76 was resolved from free [^{32}P] A76 by thin layer chromatography (TLC) and then quantified using a BioRad model FX Phosphorimager.

ATP hydrolysis experiments

These assays were performed as described previously (6). Briefly, the reactions were carried out at 37 °C in Buffer A with 3 mM [γ-^{32}P] ATP, 2 μM enzyme (wild type ThrRS or ΔN1N2 ThrRS) (27), 0.02 U/mL PPase in the presence or absence of 3′-dA76 tRNA. Reactions contained threonine at 5 mM and/or β-hydroxynorvaline at 375 mM. Ten μL reaction aliquots were quenched in 7% perchloric acid and activated charcoal solution. The quenched reaction mixture was vortexed followed by centrifugation for 3 min at 12,000 rpm, and then the amount of [γ-^{32}P] PPi in 100 μL of supernatant was determined by scintillation counting. The background rate of ATP hydrolysis in the absence of enzyme was subtracted from all determinations.

Pre-steady state kinetics

All the experiments were performed at 37 °C and pH 8, as described in prior publications (24,28). The multiple turnover reactions were performed in a rapid chemical quench flow device (RQF-3, KinTek, Austin, TX) in Buffer A by rapidly mixing 2 μM wild type ThrRS, 8 μM [^{32}P] $tRNA^{Thr}$ with 2.5 mM ATP, 40 mM HNV. At various time points, the reactions were terminated in a quench solution consisting of 3 M sodium acetate (pH 5). The

quenched product was immediately added to chilled absolute ethanol, and then allowed to precipitate for 2 hrs at −20 °C. The product was collected by centrifugation, re-suspended in buffer containing S1 nuclease, processed, and analyzed thin layer chromatography. Similar reactions were employed in the determination of the rate of AMP formation in the presence or absence of tRNA, except that 1 μM wild type ThrRS, 100 μM [α-^{32}P] ATP, and 6 μM $tRNA^{Thr}$ was used. In both the multiple turnover and AMP formation assays, the data was analyzed by linear regression. Under single turnover conditions, HNV adenylate was pre-formed by mixing 40 μM wild type ThrRS, 2.5 mM ATP, and mM HNV in buffer A for 30 min followed by removal of unincorporated substrates through the gel-filtration columns. The ThrRS:HNV-AMP complex was then rapidly mixed with 4 μM $tRNA^{Thr}$, with reaction aliquots being removed at various time points thereafter to generate a first order progress curve (25). The plot of product versus time was fit to a single exponential equation,

$$y=A^{*}(1-exp^{-k^{*}t})+C$$

where A represents the amplitude of exponential phase, *k* is the rate of burst phase, and *C* is the y-offset.

Bacterial growth experiments

A 2 mL culture of tetracycline-resistant *E. coli* was prepared in Luria-Bertani media (LB) supplemented with 10 μg/mL tetracycline, and then grown overnight at 37 °C. This culture was then used to inoculate 3 mL minimal media (1x MOPS minimal medium mixture, 1.32 mM K_2HPO_4, 1.0 % glucose, 10 μg/mL tetracycline, and 20 μg/mL of each standard amino acid) cultures with and without exogenous threonine, providing a starting OD_{600} of ~0.04. HNV was added to cultures containing and lacking threonine to final concentrations of 0, 5, or 20 mM. The cultures were placed at 37 °C, and then growth was monitored every 45 min by measurement of OD_{600}.

Purification and analysis of HNV-substituted thioredoxin

Thioredoxin (Trx) was produced from an *E. coli* overproducing strain kindly provided by Dr. Robert Hondal (University of Vermont). A total of six liters of an LB amp culture of the overproducer strain were grown to an OD_{600} of 1.0 at 37 °C, and then induced with IPTG to a final concentration of 0.5 mM. Two flasks (2 × 1.5 L) were seeded with HNV (dissolved in 20 mM Tris EDTA) to a final concentration of 1.5 mM, while the other two cultures received buffer only. The cultures were then transferred to 16 °C, and then incubated for overnight for an additional 16 hours. The purification of both natural and HNV-substituted thioredoxin was accomplished according to a previously published protocol (29). Briefly, the purification protocol consists of cell disruption by sonication, chromatography on DEAE-Sephacel (Sigma-Aldrich), precipitation by 60% ammonium sulfate saturation, and final chromatography on Sephacryl S-100 (GE Health Sciences). Fractions containing thioredoxin were identified by electrophoresis on 15% SDS-PAGE. The thioredoxin concentration was calculated using an extinction coefficient (A_{280}) of 13,700 M^{-1} cm^{-1}. The essentially (> 95%) pure fractions were pooled, dialyzed into 20 mM ammonium bicarbonate, and then lyophilized to dryness. The lyophilized samples were then submitted to N-terminal protein sequencing and mass spectroscopy. The N-terminal amino acid sequence (first ten residues) was analyzed by automatic Edman degradation using an Applied Biosystems Procise 494/HT protein sequencer (Applied Biosystems, Foster City, CA, USA). The sequencing was performed at the Protein Chemistry Laboratory of the University of Texas Medical Branch (Galveston, TX, USA).

The liquid chromatography mass spectrometry (LC/MS) analysis was performed essentially as described in (30). Prior to treatment with trypsin (20 ng/μL), cysteines in the Trx preparations were reduced and blocked by incubation with iodoacetamide under reducing conditions. The reactions were terminated with a small amount of 10% formic acid, and then subjected to electrospray ionization (ESI) LC/MS, employing a fused-silica microcapillary MagicC18 LC column (12 cm × 100 μM i.d.). The ESI instrument was attached to a LTQ linear quadrupole ion trap-Orbitrap mass spectrometer (Thermo Electron, San Jose, CA). This latter instrument was operated in collisional-induced dissociation mode to allow collection of both MS and MS/MS spectra. The chromatography of the tryptic peptides was achieved by use of a 5–50% acetonitrile gradient in 0.1% formic acid, operating at a flow rate of 0.25 μl/min. Following acquisition of the full MS scan in the data-dependent acquisition mode, the 10 most abundant ions were subjected to MS/MS analysis. The spectra obtained were then processed using SEQUEST (Bioworks software package, version 3.3.1, Thermo Electron, San Jose, CA).

RESULTS

ThrRS exhibits nearly identical catalytic specificities for threonine and β-hydroxynorvaline

HNV is a non-standard amino acid in which the characteristic methyl group of the threonine side chain is replaced by an ethyl group (Fig. 1A and B). Previously, pyrophosphate exchange experiments revealed that the k_{cat}/K_M for HNV is only 30-fold less than that of threonine (Table 1). HNV also serves as a substrate for ThrRS in the full aminoacylation reaction, but the k_{cat} and K_M parameters for this reaction are unknown (21, 31). (The lack of available radiolabeled versions of HNV may have previously precluded such experiments.) To address this issue, we employed a sensitive assay featuring $tRNA^{Thr}$ [^{32}P]-labeled at nucleotide A76 to measure aminoacylation kinetics (26, 32). Following the aminoacylation reaction, the charged tRNA was digested with S1 nuclease, and then the AA-[^{32}P]A76 and [^{32}P]A76 products were resolved by thin layer chromatography (TLC) and phosphorimaging. Notably, this assay permits high concentrations of amino acids to be used, mitigating the specific activity issues accompanying use of radioactive amino acids. The apparent k_{cat} of HNV (0.4 s^{-1}) was within two-fold of the apparent k_{cat} value obtained for threonine (0.2 s^{-1}), while the K_M of HNV was increased 38-fold, relative to threonine. Based on the overall aminoacylation efficiency (k_{cat}/K_M), the specificity of ThrRS for HNV is only 20 fold less than that of threonine, which is actually less than the level of discrimination (30-fold) measured in pyrophosphate exchange experiments (Fig. 1C and 1D). Thus, measurements of steady state kinetic parameters suggest that ThrRS does not significantly discriminate between cognate threonine and HNV during the aminoacylation reaction.

Amino acid activation is the potential rate-limiting step of HNV aminoacylation

For a number of class II ARSs, such as HisRS and ThrRS, the rate-determining step for the overall aminoacylation reaction in the presence of cognate amino acid is amino acid activation (24,28). To determine whether this attribute is characteristic of HNV, pre-steady state experiments were performed by application of the rapid chemical quench method. Under multiple turnover conditions, a linear plot of product formation was observed with an apparent k_{cat} value of 1 s^{-1} (Fig. 2A), approximately 2.5-fold greater than the value obtained from steady state aminoacylation (0.4 s^{-1}). No burst or lag phase was seen, indicating that neither product release nor substrate binding are likely to be the rate limiting step. The apparent rate of aminoacyl transfer (k_{trans}) was determined in a single turnover experiment in which excess preformed ThrRS:HNV-AMP was rapidly mixed with [^{32}P] $tRNA^{Thr}$. The value of k_{trans} (Fig. 2B) obtained was 10.4 s^{-1}, which is intermediate between the k_{trans} values for serine (7.8 s^{-1}) and threonine (14.5 s^{-1}) determined previously (6,24). Coupled

with the absence of burst kinetics, the fast rate of transfer relative to the rate of multiple turnover aminoacylation suggests that activation may be the likely rate-determining step for HNV, as it is for cognate threonine.

HNV stimulates ATPase activity in the active site of ThrRS

We previously observed excess ATP hydrolysis in the presence of near-cognate standard amino acid serine (6). To test for similar behavior with HNV, ATP hydrolysis experiments were conducted using [γ-^{32}P] ATP, monitoring the formation of [^{32}P] PPi. The rate of PPi formation by ThrRS in the presence of HNV was 12-fold higher than that observed with threonine (0.5 min^{-1} versus 0.04 min^{-1}), suggesting that HNV stimulates ATPase activity in ThrRS (Fig. 3A). In some models, the site of ATPase activity has been attributed to the editing site, which is located in a domain separate from the canonical aminoacylation active site (33,34). To test this hypothesis, HNV-stimulated ATPase assays were performed with a post-transfer editing deficient variant of ThrRS (ΔN1N2 ThrRS). The rate of HNV-stimulated PPi production (0.2 min^{-1}) with editing-deficient ThrRS was within 3 fold of that of the wild type enzyme (0.5 min^{-1}) (Fig. 3B), suggesting that ATPase activity is confined to the aminoacylation active site. The ATPase activity was not stimulated by non-aminoacylatable 3′-deoxy A76 tRNA (Fig. 3C), showing that this activity for HNV is tRNA-independent, as it is for serine (6).

HNV is not efficiently edited by ThrRS

The experiments above indicated that, like the editing substrate serine, HNV elicits ATP hydrolysis above the level seen with threonine. *A priori*, this is suggestive of pre-transfer editing. This issue was investigated by measuring the rate of [α-^{32}P] AMP formation under pre-steady state conditions in the presence and absence of tRNA. The rates of AMP formation in the presence and absence of tRNA were essentially identical (Fig 4A), suggesting that most of the adenylate formed is associated with a productive transfer of amino acid to $tRNA^{Thr}$. This observation is consistent with previous measurements of the rate of AMP formation in presence of serine, under conditions where post-transfer editing is abolished by an editing site mutation (6). Additional measurements of the rate of the enzyme-independent hydrolysis of HNV-adenylate (Fig. 4B) showed that it is much slower than the rate of AMP formation (0.001 s^{-1} versus 2.9 s^{-1}). This observation strongly suggests that the AMP formed is due to the hydrolysis of HNV adenylate in the active site of ThrRS, rather than after adenylate release and solution hydrolysis. Thus, pre-transfer editing is unlikely to occur in the presence of tRNA, which is similar to the conclusions from the investigation of serine (6).

The deacylation rate of Ser-$tRNA^{Thr}$ by wild type ThrRS was previously determined by transient kinetic experiments to be in the range of 21 s^{-1} (35). On the basis of published structures, the editing site of ThrRS readily accommodates serine but not threonine (36). As yet, the ability of the editing site to accommodate HNV has not been tested. The rate of ThrRS-dependent deacylation of HNV-[^{32}P]$tRNA^{Thr}$ was measured in rapid chemical quench experiments (Fig. 4C). In parallel, the background rate of hydrolysis in solution was also determined (Fig. 4D). These experiments showed that the rate of ThrRS-mediated deacylation was essentially no faster than the rate of deacylation by solvent (0.0003 s^{-1} versus 0.0004 s^{-1}). Significantly, both rates are 70,000 fold slower than the rate of Ser-$tRNA^{Thr}$ deacylation by wild type ThrRS. Thus, ThrRS does not deacylate HNV-$tRNA^{Thr}$, which may be a consequence of the inability of the unnatural amino acid to be accommodated by the editing site.

HNV competes with threonine and is toxic for bacteria

With the exception of the additional side chain methyl group, HNV is chemically identical to threonine. The binding pocket for threonine in the ThrRS aminoacylation active site appears to lack the close steric complementarity with the threonine substrate side chain that would be required to preclude HNV binding, accounting for its ready activation (37). If these assumptions are correct, then one would expect that HNV would be able to compete with threonine at the level of binding and aminoacyl transfer. To test this possibility, aminoacylation reactions with wild type ThrRS were executed with 100 μM [^{14}C] threonine as amino acid substrate, in the presence of increasing concentrations of HNV. As the concentration of HNV increased, the plateau levels of Thr-tRNA^{Thr} formation gradually decreased (Fig. 5A). At 0.2 mM and 2 mM concentrations of HNV, the plateau levels were decreased by 20% and 80%, respectively. At 10 mM HNV, Thr-tRNA^{Thr} formation was virtually abolished, suggesting that both amino acids are competing for the same active site.

Previous work indicates that HNV can be readily incorporated into peptides in cell free systems (31), consistent with the observations above that ThrRS readily accepts HNV as a substrate during aminoacylation and lacks editing mechanisms to prevent its attachment to cognate tRNA. Were HNV to be incorporated into proteins *in vivo*, it might well be expected to have negative consequences for bacterial growth. This hypothesis was tested in experiments that followed *E. coli* growth (as measured by an increase in OD_{600}) in the presence of threonine and increasing concentrations of HNV. A sigmoidal growth curve was obtained in the absence of any amino acid, similar to the curve obtained in the presence of threonine (Fig. 5B). However, the presence of 5 mM HNV in the culture medium resulted in more than a 90% reduction in the rate of growth. When 1 mM threonine was also included in the medium, the plateau level was lower than in the absence of threonine. Similar results were obtained at 20 mM HNV concentration in the presence or absence of 1 mM threonine (Fig. 5B and 5C). These *in vivo* bacterial experiments clearly suggest that HNV exhibits inhibitory effects on bacterial growth under standard conditions.

Incorporation of HNV into cellular proteins

The toxicity of HNV to bacterial growth in minimal media supplemented with amino acids may reflect the production of proteins with compromised structures, owing unacceptable levels of unnatural amino acid incorporation. Alternatively, undetermined mechanisms of inhibition may exist whereby HNV blocks physiological pathways essential for growth. In order to test the first model, a bacterial strain that overproduces the small reporter protein thioredoxin (Trx) was grown in rich media in the presence or absence of 1.5 mM HNV, a value that approaches the K_M for HNV in the pyrophosphate exchange reaction catalyzed by ThrRS (Table 1). Trx from these two cultures was purified in parallel to homogeneity, and then subjected to Edman N-terminal sequencing, as well as trypsin digestion followed by ESI-LC/MS. The Trx preparations obtained by the sequence of DEAE-Sepharose and S-100 Sephacryl chromatographies was approximately > 95% pure (fig. S1). Trx has six threonines, distributed among five of ten peptides detectable by LC/MS (i.e., greater than 500 Daltons). (Mass spectrometry coverage included all peptides containing threonine.) Residue nine of the Trx protein (not counting the methionine removed from the N-terminus) is a threonine, so the first approach undertaken to look for incorporation was N-terminal (Edman Degradation) sequencing of the first ten residues of the protein (S^{1}DKIIHLTD^{10}). Although initial analyses of HNV-supplemented Trx gave an extra peak (retention time (RT) = 5.83–5.85 min) in addition to threonine that was tentatively assigned to HNV (fig S2), further analysis of both the non-supplemented Trx, a control protein with Thr at position, and chromatography of an authentic PTH-HNV standard indicated that this is more likely to be breakdown product of PTH-Thr (fig S2). This conclusion gained support from the observed retention time of PTH-HNV, 6.17 min (fig S2).

In order to gain information about potential substitutions at the other positions, the HNV-supplemented and non HNV-supplemented Trx preparations were also subjected to trypsinolysis and LC/MS. These experiments showed that one for one of the threonine-containing peptides (L^{59}NIDQNPGTAPK^{70}), a subpopulation could be detected that was 14 mass units greater than 1267.6641 Da predicted for the peptide that contained threonine (Thr-67) (Figure 6A). Significantly, this subpopulation was absent in the corresponding peptide from the Trx prepared in the absence of HNV (Figure 6B). These data suggest that, when *E. coli* is grown in the presence of sub-toxic concentrations of HNV, it is possible to detect the presence of the unnatural amino acid in a reporter protein.

DISCUSSION

Aminoacyl-tRNA synthetases evolved to address discrimination issues between the 20 cognate standard amino acids

ARSs are highly specific for the selection of substrates, and mistakes in aminoacylation are corrected by editing mechanisms (3). When substrate specificity and editing mechanisms of ARSs are relaxed, mistranslation occurs, potentially affecting cellular physiology (38–42). During the evolution of the ARSs and other components of the translational apparatus, strong selection pressures were likely in effect to prevent mis-incorporation of amino acids that are smaller than the cognate. Owing to the fact that not all amino acids have closely related neighbors in chemical space, editing functions are limited to those ARS that face significant amino acid discrimination problems, including IleRS, ValRS, LeuRS, MetRS, ThrRS, ProRS, PheRS, AlaRS. For each of these, there exists at least one other near cognate amino acid that is activated to a significant extent by the editing ARS. Until recently, efforts have focused on how the enzymes favor the cognate amino acid and discriminate against the naturally occurring non-cognates (3,10,43). While studies of the discrimination between naturally occurring amino acids can provide insights into how errors in normal protein synthesis are avoided, they are unable to provide a complete picture of the total substrate recognition properties of the enzymes, particularly the ‘chemical space’ that may lie outside the 20 canonical amino acids.

HNV evades the normal ThrRS editing mechanism

In general, the discrimination threshold (based on $[(k_{cat}/K_{M]cognate})/(k_{cat}/K_{M]noncognate})]$ for a requirement for editing is conventionally taken to be in the neighborhood of 1 error in 2,000. Owing to a specificity constant that is within three orders of magnitude of that of cognate threonine, serine therefore poses a significant challenge to the fidelity of ThrRS (6,37). ThrRS addresses this challenge by the combined screening effects of the aminoacylation and the editing active sites, both of which can potentially contribute to editing. In the aminoacylation site, a coordinated zinc provides specificity for polar, hydroxyl-containing amino acids like threonine and serine, but actively discriminates against valine and any smaller non-polar amino acid (13,37). The aminoacylation active site readily accepts serine, which is also the preferential substrate for the editing site (37). Notably, the selectivity of the aminoacylation active site is principally dictated at the level of binding; once adenylate is formed; threonyl- and seryl-adenylate undergo transfer to tRNA at similar rates (6). There is no discrimination at the level of chemistry. Recent work showed that editing of serine occurs mostly through post-transfer editing, and that pre-transfer editing is important only when the rate of aminoacyl transfer is slowed significantly (6). Thus, the rate of aminoacyl transfer appears to dictate the relative contributions of the two different pathways. Operationally, the post-transfer editing site is thus the critical site for providing selectivity for serine over threonine. Nevertheless, how the editing site achieves full specificity for serine over threonine is not fully understood (36).

Previously, HNV was shown to be a substrate for ThrRS, by virtue of a mass-spectroscopy based incorporation assay, and by virtue of its incorporation in peptides (21,31). With the exception of the initial binding step, our studies showed that HNV appears to participate in the elementary steps of aminoacylation with kinetics that are essentially equal to that of threonine (Figure 1 and Table 1). The reduced affinity for the ThrRS aminoacylation active site leads to an increased K_M that corresponds to a 1.7–2.0 kcal reduction in binding energy. Notably, this is much smaller than the 740-fold decrease in affinity observed with serine, which is equivalent to 2.9 kcal. The presence of a β-methylene group in the side chain appears to be necessary for high affinity binding, but the active site readily allows the longer aliphatic chain of HNV to be tolerated. While serine and HNV differ markedly in their ability to elicit editing function in ThrRS, the property they share that diverges from cognate threonine is their stimulation of a latent ATPase activity in the ThrRS aminoacylation site (Figure 3) . This suggests that mis-positioning of amino acids in the synthetic site, (which would be reflected in an increase in the K_M parameter) is also correlated with increased ATPase levels, even when the amino acid is not subjected to editing. Accordingly, screening for amino acid-induced ATPase activity may be useful in an initial rapid scan of compound libraries to find those with appreciable affinity for the enzyme active site.

These results indicate that, were HNV to be a part of normal cellular physiology, then ThrRS would likely have evolved the ability to discriminate against it. HNV, however, only appears in bacterial physiology under the unusual condition when pyridoxine auxotrophs of *E. coli* are starved for pyridoxine (44). By contrast, ValRS is obliged to discriminate under standard physiological conditions amino acid against isoleucine, which (like HNV) is but one methyl group larger than the cognate. The specificity constant of ValRS (V_{max}/K_M) for isoleucine is 7.5×10^{-5} relative to that of cognate valine (45), representing a level of discrimination many orders of magnitude greater than the discrimination against HNV by ThrRS. Thus, there is considerable biological selection pressure for ValRS to discriminate against isoleucine during metabolism, while virtual none for ThrRS to discriminate against HNV. This underscores the degree to which the specificity properties of ARSs are shaped by evolutionary pressures.

Fidelity escape and the general problem of incorporating unnatural amino acids

Much of the work on UAA can be traced to early observations that fluorinated phenylalanines and hydroxyl tryptophans are readily accepted as substrates by ARSs, and can be incorporated into proteins. An important advance was the identification of a special PheRS mutant, A294G, with reduced ability to discriminate against fluoro-phenylalanine (46,47). The activation of near-cognate non-standard amino acids was subsequently shown to be quite general, and characteristic of many ARS systems. Class II ProRS misactivates *cis*- and *trans*-4-hydroxyproline, and azetidine (48); AlaRS misactivates aminobutyrate (49); SerRS misactivates serine hydroxamate and 3-aminoalanine (50); and LysRS misactivates homocysteine, homoserine, and ornithine (51). Among the Class I ARSs, LeuRS misactivates several amino acids including norvaline and norleucine (52). Similar to the relationship of threonine and HNV, several of these analogs differ from the cognate by a single methyl group.

More recently, the emphasis has shifted from anecdotal studies of UAA incorporation to more focused efforts to engineer ‘orthognal’ dedicated ARS-tRNA pairs able to insert particular UAA at specific sites in a protein of interest (53). While such efforts have focused mainly on TyrRS variants, the range of different UAAs incorporated is quite impressive (18,54). An important consequence of the directed efforts is that they have served to raise general interest in the problem of unnatural amino acid incorporation, including a deeper appreciation of the actual potential of ARSs to promote promiscuous aminoacylation. In one of the first efforts to systematically explore the ‘chemical space’ around aminoacylation,

Josephson (31) tested twelve different UAAs, and found that all could be readily aminoacylated, and incorporated into peptides by cell free protein synthesizing systems. The 12 UAAs, which included HNV, represented substrates that were equally distributed among class I and class II ARSs. Notably, a number of the UAAs had chemical properties distinct from their parent amino acids, including substantially different pKa's and other properties. In follow up studies, Hartman *et al.* examined > 190 different commercially available UAAs, and determined that at least 90 can be functionally charged on to tRNAs (21,55). The main conclusion from this work is that ARSs that charge non-polar amino acids appear to possess a broader "chemical space" with respect amino acid substrates around than the ARSs that aminoacylate the smaller and more polar amino acids. MetRS appears to be particularly promiscuous. "Fidelity escape" thus appears to be common to many of the synthetases, and not just those that have editing functions. It is likely that further explorations of the 'chemical space' revealed by these experiments will provide the shortest path to the design of stable, engineered ARSs that are able to incorporate the most useful derivatives.

The cellular toxicity associated with HNV

The mass spectrometry-based aminoacylation assay and peptide incorporation assay collectively serve to highlight those UAAs for which the standard protein synthesizing system poses no specific barrier to efficient incorporation (55). Such systems should be readily applicable to the development of peptide reagents with multiple UAAs, imparting to them useful properties (21,55). A key feature of the UAA identified in the recently described studies is that they do not provide any information about the effect of the UAA on global cellular physiology, including information about uptake or global protein synthesis. Here, we found that the ability of ThrRS to aminoacylate HNV and presumably incorporate it into proteins is accompanied by significant toxicity in minimal media. This toxicity could be decreased to acceptable levels by decreasing the concentration of the unnatural amino acid, and by growth in rich media. Under these conditions, the very low levels of incorporations may preclude detection of an effect on the growth kinetics. When incubated at higher concentrations, HNV may affect protein post-translational modifications, particularly in animal cells. An important caveat is that none of the experiments reported here address potential "off-target" effects of HNV, i.e. acting as an inhibitor of unidentified enzymes or pathways essential for growth. In mammalian cells, toxic effects of HNV appear to include impairment of the folding and export of collagen (23,56), inhibition of O- and N-glycosylation of glycoproteins (57), and teratogenic effects on chicken and mouse embryos (58). These observations correlate with the lowered bacterial growth in this study. Thus, HNV is toxic to both mammalian and bacterial cells. Additional studies are required to determined whether or not toxicity is a general property of all amino acid analogs that are subject to fidelity escape, or merely those with deleterious effects on protein structure or function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS

AlaRS	alanyl-tRNA synthetase
AMP	adenosine monophosphate
ARS	aminoacyl-tRNA synthetase

HNV	β-hydroxynorvaline
CP1	connective polypeptide one [from class I aminoacyl-tRNA synthetases]
ESI	electrospray ionization
LeuRS	leucyl- tRNA synthetase
LB	Luria-Bertani media
LC/MS	liquid chromatography mass spectrometry
LysRS	lysyl-tRNA synthetase
MetRS	methionyl- tRNA synthetase
MOPS	3-(N-morpholino)propanesulfonic acid
MS	mass spectrometry
PAGE	polyacrylamide gel electrophoresis
PheRS	phenylalanyl- tRNA synthetase
ProRS	prolyl-tRNA synthetase
PPi	pyrophosphate
PPase	pyrophosphatase
PTH-HNV	phenylthiohydantoin-derivatized hydroxynorvaline
SerRS	seryl-tRNA synthetase
ThrRS	threonyl- tRNA synthetase
TCA	trichloroacetic acid
Trx	thioredoxin
UAA	unnatural amino acids
ValRS	valyl-tRNA synthetase

Acknowledgments

We thank Robert Hondal and Adam Lothrup for the Trx overproducing strain and assistance in its purification, Steve Smith of the Protein Chemistry Laboratory of the University of Texas Medical Branch for N-terminal protein sequencing, Dallin Bagley and Amy Rumora for contributions during the initial phases of this study, and Adam Mirando for comments on the manuscript.

REFERENCES

1. Reynolds NM, Lazazzera BA, Ibba M. Cellular mechanisms that control mistranslation. Nat Rev Microbiol. 2010; 8:849–856. [PubMed: 21079633]
2. Ibba M, Soll D. Aminoacyl-tRNA synthesis. Ann. Rev. Biochem. 2000; 69:617–650. [PubMed: 10966471]
3. Francklyn CS. DNA polymerases and aminoacyl-tRNA synthetases: shared mechanisms for ensuring the fidelity of gene expression. Biochemistry. 2008; 47:11695–11703. [PubMed: 18850722]
4. Pauling, L. The probability of errors in the process of synthesis of protein molecules. In: Birkhauser, A., editor. Festschrift fur Prof. Dr. Arthur Stoll. Basel, Switzerland: Birkhauser Verlag; 1958. p. 597-602.
5. Fersht, AR. Specificity and Editing Mechanisms. In: Fersht, AR., editor. Structure and Mechanism in Protein Science. New York: WH Freeman; 1999.

6. Minajigi A, Francklyn CS. Aminoacyl transfer rate dictates choice of editing pathway in threonyl-tRNA synthetase. J. Biol. Chem. 2010; 285:23810–23817. [PubMed: 20504770]
7. Dulic M, Cvetesic N, Perona JJ, Gruic-Sovulj I. Partitioning of tRNA-dependent editing between pre- and post-transfer pathways in class I aminoacyl-tRNA synthetases. J. Biol. Chem. 2010; 285:23799–23809. [PubMed: 20498377]
8. Boniecki MT, Vu MT, Betha AK, Martinis SA. CP1-dependent partitioning of pretransfer and posttransfer editing in leucyl-tRNA synthetase. Proc. Natl. Acad. Sci. USA. 2008; 105:19223–19228. [see comment]. [PubMed: 19020078]
9. Tan M, Zhu B, Zhou XL, He R, Chen X, Eriani G, Wang ED. tRNA-dependent pre-transfer editing by prokaryotic leucyl-tRNA synthetase. J. Biol. Chem. 2009; 285:3235–3244. [PubMed: 19940155]
10. Mascarenhas, A.; Martinis, S.; An, S.; Rosen, AE.; Musier-Forsyth, K. Fidelity Mechanisms in Aminoacyl-tRNA Synthetases. In: RajBhandary, UL.; Koehrer, C., editors. Protein Engineering. New York, NY: Springer-Verlag; 2008.
11. Jakubowski H, Fersht AR. Alternative pathways for editing non-cognate amino acids by aminoacyl-tRNA synthetases. Nucl. Acids Res. 1981; 9:3105–3117. [PubMed: 7024910]
12. Crepin T, Yaremchuk A, Tukalo M, Cusack S. Structures of two bacterial prolyl-tRNA synthetases with and without a cis-editing domain. Structure. 2006; 14:1511–1525. [PubMed: 17027500]
13. Sankaranarayanan R, Dock-Bregeon A-C, Romby P, Caillet J, Springer M, Rees B, Ehresmann C, Ehresmann B, Moras D. The structure of threonyl-tRNA synthetase-tRNA^{Tyr} complex enlightens its repressor activity and reveals an essential zinc ion in the active site. Cell. 1999; 97:371–381. [PubMed: 10319817]
14. Naganuma M, Sekine S, Fukunaga R, Yokoyama S. Unique protein architecture of alanyl-tRNA synthetase for aminoacylation, editing, and dimerization. Proc. Natl. Acad. Sci. U.S.A. 2009; 106:8489–8491. [PubMed: 19423669]
15. Sokabe M, Ose T, Nakamura A, Tokunaga K, Nureki O, Yao M, Tanaka I. The structure of alanyl-tRNA synthetase with editing domain. Proc. Natl. Acad. Sci. U. S. A. 2009; 106:11028–11033. [PubMed: 19549823]
16. Mosyak L, Reshetnikova L, Goldgur Y, Delarue M, Safro MG. Structure of phenylalanyl-tRNA synthetase from *Thermus thermophilus*. Nature Struct. Biol. 1995; 2:537–547. [PubMed: 7664121]
17. Ling J, Roy H, Ibba M. Mechanism of tRNA-dependent editing in translational quality control. Proc. Natl. Acad. Sci. USA. 2007; 104:72–77. [PubMed: 17185419]
18. Liu CC, Schultz PG. Adding new chemistries to the genetic code. Annu Rev Biochem. 2010; 79:413–444. [PubMed: 20307192]
19. Doring V, Mootz HD, Nangle LA, Hendrickson TL, de Crecy-Lagard V, Schimmel P, Marliere P. Enlarging the amino acid set of Escherichia coli by infiltration of the valine coding pathway. Science. 2001; 292:501–504. [PubMed: 11313495]
20. Tang Y, Tirrell DA. Attenuation of the editing activity of the Escherichia coli leucyl-tRNA synthetase allows incorporation of novel amino acids into proteins in vivo. Biochemistry. 2002; 41:10635–10645. [PubMed: 12186549]
21. Hartman MC, Josephson K, Szostak JW. Enzymatic aminoacylation of tRNA with unnatural amino acids. Proc. Natl. Acad. Sci. U. S. A. 2006; 103:4356–4361. [PubMed: 16537388]
22. Budisa N. Prolegomena to future experimental efforts on genetic code engineering by expanding its amino acid repertoire. Angew Chem Int Ed Engl. 2004; 43:6426–6463. [PubMed: 15578784]
23. Christner P, Carpousis A, Harsch M, Rosenbloom J. Inhibition of the assembly and secretion of procollagen by incorporation of a threonine analogue, hydroxynorvaline. J. Biol. Chem. 1975; 250:7623–7630. [PubMed: 1236848]
24. Minajigi A, Francklyn CS. RNA-assisted catalysis in a protein enzyme: The 2'-hydroxyl of tRNA(Thr) A76 promotes aminoacylation by threonyl-tRNA synthetase. Proc. Natl. Acad. Sci. U. S. A. 2008; 105:17748–17753. [PubMed: 18997014]
25. Francklyn CS, First EA, Perona JJ, Hou YM. Methods for kinetic and thermodynamic analysis of aminoacyl-tRNA synthetases. Methods (Duluth). 2008; 44:100–118.
26. Ledoux S, Uhlenbeck OC. [3'-32P]-labeling tRNA with nucleotidyltransferase for assaying aminoacylation and peptide bond formation. Methods (Duluth). 2008; 44:74–80.

27. Dock-Bregeon A, Sankaranarayanan R, Romby P, Caillet J, Springer M, Rees B, Francklyn CS, Ehresmann C, Moras D. Transfer RNA-mediated editing in threonyl-tRNA synthetase. The class II solution to the double discrimination problem. Cell. 2000; 103:877–884. [PubMed: 11136973]
28. Guth E, Connolly SH, Bovee M, Francklyn CS. A substrate-assisted concerted mechanism for aminoacylation by a class II aminoacyl-tRNA synthetase. Biochemistry. 2005; 44:3785–3794. [PubMed: 15751955]
29. Eckenroth BE, Lacey BM, Lothrop AP, Harris KM, Hondal RJ. Investigation of the C-terminal redox center of high-Mr thioredoxin reductase by protein engineering and semisynthesis. Biochemistry. 2007; 46:9472–9483. [PubMed: 17661444]
30. Thornton TM, Pedraza-Alva G, Deng B, Wood CD, Aronshtam A, Clements JL, Sabio G, Davis RJ, Matthews DE, Doble B, Rincon M. Phosphorylation by p38 MAPK as an alternative pathway for GSK3beta inactivation. Science. 2008; 320:667–670. [PubMed: 18451303]
31. Josephson K, Hartman MC, Szostak JW. Ribosomal synthesis of unnatural peptides. J. Am. Chem. Soc. 2005; 127:11727–11735. [PubMed: 16104750]
32. Uter NT, Gruic-Sovulj I, Perona JJ. Amino acid-dependent transfer RNA affinity in a class I aminoacyl-tRNA synthetase. J. Biol. Chem. 2005; 280:23966–23977. [PubMed: 15845537]
33. Nomanbhoy TK, Hendrickson TL, Schimmel P. Transfer RNA-dependent translocation of misactivated amino acids to prevent errors in protein synthesis. Mol Cell. 1999; 4:519–528. [PubMed: 10549284]
34. Silvian LF, Wang J, Steitz TA. Insights into editing from an ile-tRNA synthetase structure with tRNAile and mupirocin. Science. 1999; 285:1074–1077. [PubMed: 10446055]
35. Waas WF, Schimmel P. Evidence that tRNA synthetase-directed proton transfer stops mistranslation. Biochemistry. 2007; 46:12062–12070. [PubMed: 17924654]
36. Dock-Bregeon AC, Rees B, Torres-Larios A, Bey G, Caillet J, Moras D. Achieving error-free translation; the mechanism of proofreading of threonyl-tRNA synthetase at atomic resolution. Mol. Cell. 2004; 16:375–386. [PubMed: 15525511]
37. Sankaranarayanan R, Dock-Bregeon AC, Rees B, Bovee M, Caillet J, Romby P, Francklyn CS, Moras D. Zinc ion mediated amino acid discrimination by threonyl-tRNA synthetase. Nature Structural Biology. 2000; 7:461–465. [see comment].
38. Nangle LA, De Crecy Lagard V, Doring V, Schimmel P. Genetic code ambiguity. Cell viability related to the severity of editing defects in mutant tRNA synthetases. J. Biol. Chem. 2002; 277:45729–45733. [PubMed: 12244062]
39. Pezo V, Metzgar D, Hendrickson TL, Waas WF, Hazebrouck S, Doring V, Marliere P, Schimmel P, De Crecy-Lagard V. Artificially ambiguous genetic code confers growth yield advantage. Proc. Natl. Acad. Sci. USA. 2004; 101:8593–8597. [PubMed: 15163798]
40. Bacher JM, Waas WF, Metzgar D, de Crecy-Lagard V, Schimmel P. Genetic code ambiguity confers a selective advantage on Acinetobacter baylyi. Journal of Bacteriology. 2007; 189:6494–6496. [PubMed: 17616603]
41. Chong YE, Yang XL, Schimmel P. Natural homolog of tRNA synthetase editing domain rescues conditional lethality caused by mistranslation. J. Biol. Chem. 2008; 283:30073–30078. [PubMed: 18723508]
42. Lee JW, Beebe K, Nangle LA, Jang J, Longo-Guess CM, Cook SA, Davisson MT, Sundberg JP, Schimmel P, Ackerman SL. Editing-defective tRNA synthetase causes protein misfolding and neurodegeneration. Nature. 2006; 443:50–55. [see comment]. [PubMed: 16906134]
43. Ling J, Reynolds N, Ibba M. Aminoacyl-tRNA Synthesis and Translational Quality Control. Annual Review of Microbiology. 2009; 63:612–678.
44. Hill RE, White RL, Smith KC. The identification of 5-hydroxy-L-norvaline in cultures of pyridoxine auxotrophs of Escherichia coli B. J Nat Prod. 1993; 56:1246–1254. [PubMed: 8229010]
45. Tardif KD, Liu M, Vitseva O, Hou YM, Horowitz J. Misacylation and editing by Escherichia coli valyl-tRNA synthetase: evidence for two tRNA binding sites. Biochemistry. 2001; 40:8118–8125. [PubMed: 11434781]
46. Kast P, Hennecke H. Amino acid Substrate Specificity of *Escherchia coli* Phenylalanyl-tRNA synthetase altered by Distinct Mutations. J. Mol. Biol. 1991; 222:99–124. [PubMed: 1942071]

47. Ibba M, Kast P, Hennecke H. Substrate Specificity is Determined by Amino acid Binding Pocket Size in *Escherichia coli* Phenylalanyl-tRNA Synthetase. Biochemistry. 1994; 33:7107–7112. [PubMed: 8003476]
48. Beuning PJ, Musier-Forsyth K. Hydrolytic editing by a class II aminoacyl-tRNA synthetase. Proc Natl Acad Sci U S A. 2000; 97:8916–8920. [PubMed: 10922054]
49. Tsui W-C, Fersht AR. Probing the Principlesof Amino Acid Selection using the Alanyl-tRNA Synthetase from *Escherichia coli*. Nucl. Acids Res. 1981; 9:4627–4637. [PubMed: 6117825]
50. Gruic-Sovulj I, Rokov-Plavec J, Weygand-Durasevic I. Hydrolysis of non-cognate aminoacyl-adenylates by a class II aminoacyl-tRNA synthetase lacking an editing domain. FEBS Letters. 2007; 581:5110–5114. [PubMed: 17931630]
51. Jakubowski H. Misacylation of $tRNA^{Lys}$ with Noncognate Amino Acids by Lysyl-tRNA Synthetase. Biochemistry. 1999; 38:8088–8093. [PubMed: 10387054]
52. Martinis SA, Fox GE. Non-standard amino acid recognition by Escherichia coli leucyl-tRNA synthetase. Nucleic Acids Symp Ser. 1997; 36:125–128. [PubMed: 11541249]
53. Liu DR, Magliery TJ, Pastrnak M, Schultz PG. Engineering a tRNA and aminoacyl-tRNA synthetase for the site-specific incorporation of unnatural amino acids into proteins *in vivo*. Proc. Natl. Acad. Sci. USA. 1997; 94:10092–10097. [PubMed: 9294168]
54. Wang L, Xie J, Schultz PG. Expanding the Genetic Code. Annu Rev Biophys Biomol Struct. 2006
55. Hartman MC, Josephson K, Lin CW, Szostak JW. An expanded set of amino acid analogs for the ribosomal translation of unnatural peptides. PLoS One. 2007; 2:e972. [PubMed: 17912351]
56. Barile FA, Siddiqi ZE, Ripley-Rouzier C, Bienkowski RS. Effects of puromycin and hydroxynorvaline on net production and intracellular degradation of collagen in human fetal lung fibroblasts. Arch Biochem Biophys. 1989; 270:294–301. [PubMed: 2494943]
57. Arenas MI, Royuela M, Fraile B, Paniagua R, Wilhelm B, Aumuller G. Identification of N- and O-linked oligosaccharides in human seminal vesicles. J Androl. 2001; 22:79–87. [PubMed: 11191091]
58. Louw R, Potgieter HC, Vorster W. Teratogenicity of 3-hydroxynorvaline in chicken and mouse embryos. Amino Acids. 2005; 29:207–212. [PubMed: 16082502]

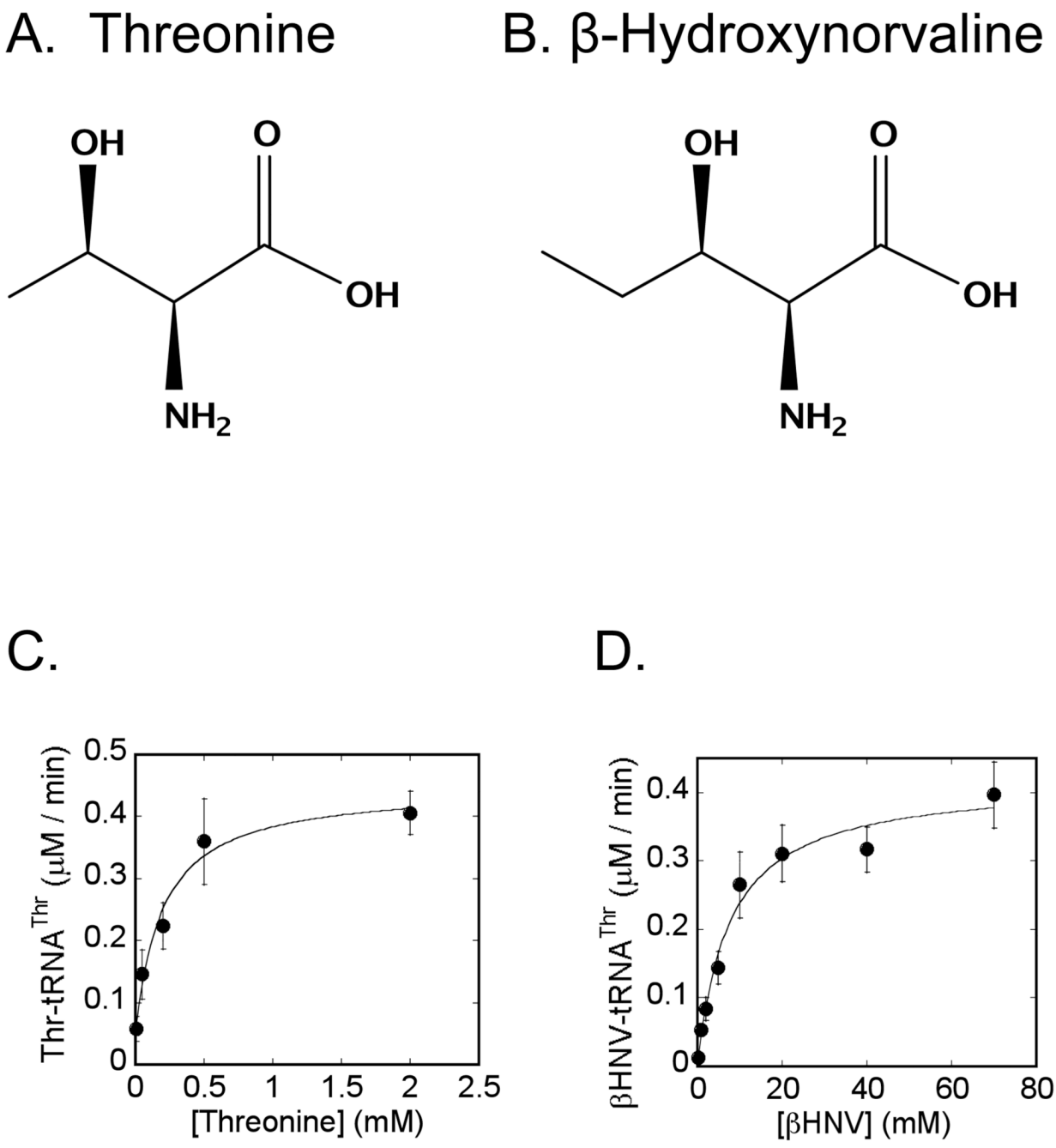

Figure 1.

β-hydroxynorvaline is readily accepted as a substrate by *E. coli* ThrRS. Chemical structures of (A) threonine and (B) β-hydroxynorvaline. Velocity versus [Amino acid] plots under steady state conditions are depicted for (C) threonine and (D) HNV. The initial rates for each velocity were determined at 37 °C and pH 8 by use of the Wolfson-Uhlenbeck assay (26). Technical details of the experiments are mentioned in the “Experimental Procedures.” Error bars represent standard errors on three independent determinations.

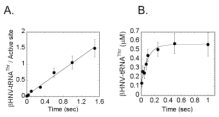

Figure 2.

Transient kinetics of HNV-tRNA^{Thr} formation by wild type ThrRS at 37 °C and pH 8. All the reactions were performed using rapid chemical quench experiments as described in "Experimental Procedures." Panel (A) depicts a multiple turnover experiment from which k_{cat} was determined by fitting to a straight line by linear regression. Panel (B) depicts a single turnover experiment in which excess preformed ThrRS bound HNV adenylate was rapidly mixed with [^{32}P] tRNA^{Thr}. The resulting first order progress curve was fit a single exponential equation, as described in "Experimental Procedures." Error bars represent standard errors on three independent experiments.

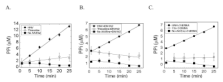

Figure 3.

ATPase activity of ThrRS in the presence of HNV at 37 °C and pH 8. All panels depict the hydrolysis of [γ-^{32}P] ATP in the presence of (A) HNV, wild type ThrRS, and no tRNA, (B) HNV, ΔN1N2 ThrRS, and no tRNA, and (C) HNV, wild type ThrRS and 3′-dA76 tRNA. The reactions were quenched at different time points, subjected to brief centrifugation (3 min at 12,000 × g), and then the [^{32}P]-PPi remaining in the supernatant was quantitated by scintilliation counting. Error bars represent standard errors on three independent determinations.

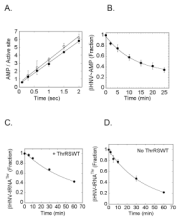

Figure 4.

Kinetic analysis of editing of HNV by ThrRS at 37 °C and pH 8. All reactions were performed using HNV as the substrate. Panel (A), linear rate of AMP formation in the presence (open circles) and absence (filled circles) of $tRNA^{Thr}$. Panel (B), enzyme-independent hydrolysis of HNV-AMP in Buffer A. Panels (C) and (D), rate of deacylation of HNV-[^{32}P] $tRNA^{Thr}$ in the presence and absence of wild type ThrRS, respectively.

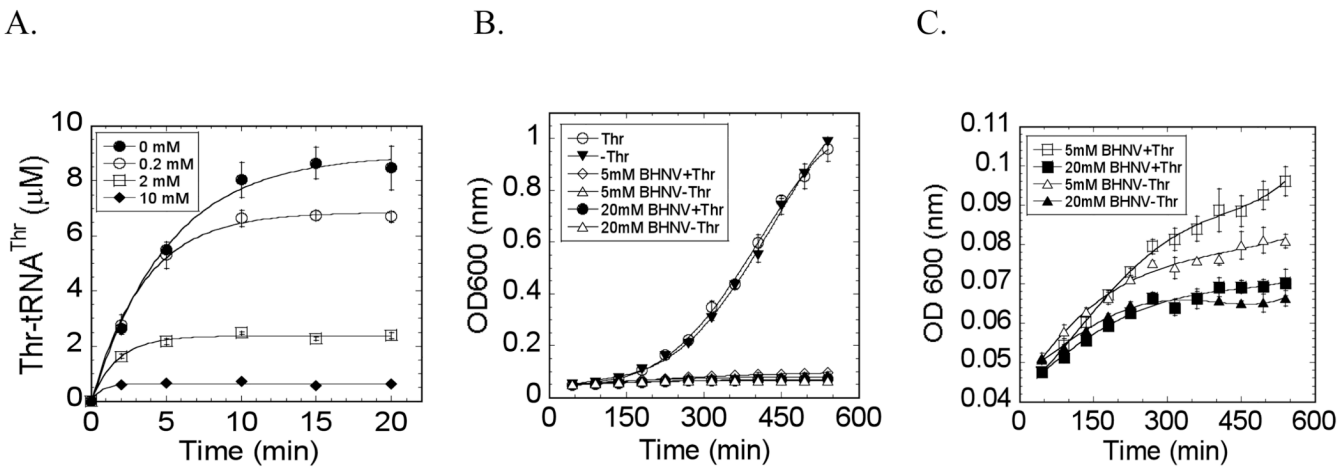

Figure 5.

Effect of HNV on aminoacylation with threonine *in vitro* and on bacterial growth *in vivo*. Panel (A), plateau levels of [^{14}C] Thr-tRNA^{Thr} formed in the absence and presence of increasing concentrations of HNV. These reactions were performed at 37 °C and pH 8. Panel (B), growth curves for *E. coli* IBPC6881 (13) grown in minimal media supplemented with either threonine or HNV as shown in the figure. Bacteria growth was measured at OD_{600} every 45 min. Panel (C) expands the y-axis of the plot in Panel B to provide a close-up of the effect of increasing HNV concentrations in the presence of threonine.

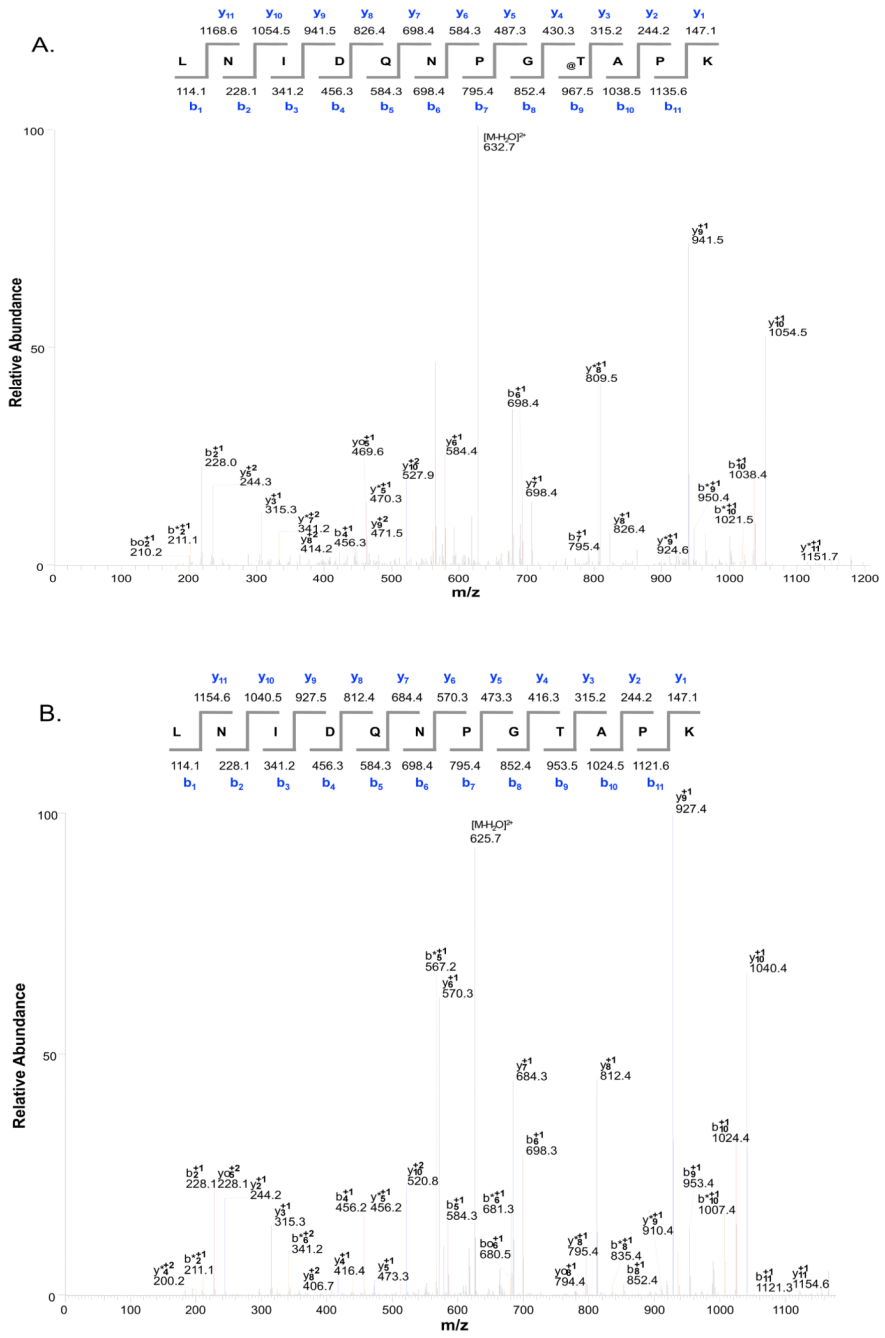

Figure 6.

ESI-MS/MS spectra of peptide LNIDQNPGTAPK in the HNV-supplemented and non HNV-supplemented thioredoxin. The marks of "*" and "o" in fragment ions represent an NH_3 loss (−17 Da) and H_2O loss (−18 Da) from their b and y-ion series, respectively. (A). The substitution of HNV into peptide of LNIDQNPGT^{67} APK. This MS/MS spectrum depicted peptide precursor ion at m/z 641.34^{2+}, which unambiguously confirmed a 14 Da mass increase on Thr-67 residue (marked with an @). (B). The peptide LNIDQNPGTAPK with precursor ion at m/z 634.43^{2+} in non HNV-supplemented thioredoxin. No increments in the molecular mass of 14 Da on Thr-67 residue has been found.

Table 1

Comparison of steady state kinetic parameters for threonine and β-hydroxynorvaline as substrates for threonyl-tRNA synthetase from *Escherichia coli*[a].

Steady state parameters	Adenylation[b] (Pyrophosphate exchange)		Aminoacylation	
	Threonine	β-hydroxy norvaline	Threonine	β-hydroxy norvaline
k_{cat} (s^{-1})	36	22	0.2 ± 0.01	0.4 ± 0.02
Km (mM)	0.11	1.95	0.2 ± 0.08	7.6 ± 2.2
k_{cat}/Km (mM^{-1} s^{-1})	327	11	1	0.05
Relative specificity	30	1	20	1

[a] All aminoacylation experiments were performed at 37 °C and pH 8.0 as described in “Experimental Procedures”. The values represent the mean ± standard error.

[b] Data previously reported in (37).