Enzymatic tRNA Acylation by Acid and α-Hydroxy Acid Analogues of Amino Acids†

Alina Owczarek,‡ Mark Safro,§ and Alexey D. Wolfson*,‡

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309, and Department of Structural Biology, Weizmann Institute of Science, 76100 Rehovot, Israel

Received July 24, 2007; Revised Manuscript Received October 24, 2007

ABSTRACT: Incorporation of unnatural amino acids with unique chemical functionalities has proven to be a valuable tool for expansion of the functional repertoire and properties of proteins as well as for structure−function analysis. Incorporation of α-hydroxy acids (primary amino group is substituted with hydroxyl) leads to the synthesis of proteins with peptide bonds being substituted by ester bonds. Practical application of this modification is limited by the necessity to prepare corresponding acylated tRNA by chemical synthesis. We investigated the possibility of enzymatic incorporation of α-hydroxy acid and acid analogues (lacking amino group) of amino acids into tRNA using aminoacyl-tRNA synthetases (aaRSs). We studied direct acylation of tRNAs by α-hydroxy acid and acid analogues of amino acids and corresponding chemically synthesized analogues of aminoacyl-adenylates. Using adenylate analogues we were able to enzymatically acylate tRNA with amino acid analogues which were otherwise completely inactive in direct aminoacylation reaction, thus bypassing the natural mechanisms ensuring the selectivity of tRNA aminoacylation. Our results are the first demonstration that the use of synthetic aminoacyl-adenylates as substrates in tRNA aminoacylation reaction may provide a way for incorporation of unnatural amino acids into tRNA, and consequently into proteins.

The ability of a translation system to incorporate unnatural amino acids is limited by the specificity of the components of the translation machinery interacting with the amino acid. While ribosome and elongation factors may affect the efficiency of the amino acid's incorporation, the utilization of amino acids in a translation system is governed by the specificity of aaRSs[1] (*1*−*3*). Several strategies have been developed to incorporate unnatural amino acids into proteins. The incorporation of unnatural amino acids *in vivo* may be achieved either by using the natural promiscuity of aaRSs (*4*, *5*) or by designing mutant aminoacyl-tRNA synthetases with altered amino acid specificity (*6*−*10*).

For *in vitro* applications, the specificity of aaRSs can be bypassed by chemically attaching the unnatural amino acid to tRNA (*11*−*13*) or by aminoacylating tRNA with the unnatural amino acid using a ribozyme system (*14*, *15*). These artificially aminoacylated tRNAs can be used for *in vitro* protein translation.

In this paper we investigated the feasibility of an alternative approach to achieve unnatural amino acid incorporation into tRNA exploiting the natural promiscuity of aaRSs. The reaction of tRNA aminoacylation is a two step process with the amino acid being first activated by ATP with formation of aminoacyl-adenylate. At the second step of reaction, amino acid is transferred from aminoacyl-adenylate to tRNA. The amino acid specificity of aminoacylation is believed to be determined by the amino acid activation step of reaction (*1*, *3*). When the discrimination of amino acid in an activation reaction is insufficient and noncognate aminoacyl-adenylate formation occurs, amino acid will be transferred to tRNA. An additional editing mechanism is involved in the correction of these errors by some aaRSs (*1*, *2*). Aminoacyl-adenylates display high affinity (nM range) to the aaRSs active sites, which exceeds affinity of both ATP and amino acid (*16*, *17*). We suggested that chemically synthesized analogues of aminoacyl-adenylates containing noncognate or unnatural amino acid may bypass the initial discrimination step by aaRS due to the additional affinity provided to adenylate by AMP. The molecular dynamic simulation of the stacking interaction between Pheα216 of *Thermus thermophilus* PheRS and the adenylate ring of Phe-AMP demonstrates the highest stability among all possible types of interactions in the environment where Phe-AMP is embedded. The stacking conformation remains unchanged during the period of 1 ns. (*18*). Consequently, any amino acid can penetrate into the amino acid binding pocket within the limits of the active site plasticity. The wild type of PheRS demonstrates a significant degree of the active site natural plasticity: side by side with cognate phenylalanine, the amino acid binding pocket can accommodate both the noncognate tyrosine and the unnatural pCl-phenylalanine (*19*). This finding agrees with the observation made by P. Shultz and coauthors: "The high degree of structural plasticity observed in aaRSs is rarely found in other mutant enzymes with altered specificities and

† This work was supported by NSF Grant MCB-0344002 to A.W.

* To whom correspondence should be addressed. Tel: 1 303 492 6088. Fax: 1 303 492 5894. E-mail: alexey.wolfson@colorado.edu.

‡ University of Colorado.

§ Weizmann Institute of Science.

[1] Abbreviations: AaRS, aminoacyl-tRNA synthetases; PheRS, phenylalanyl-tRNA synthetases; Phe-AMP, phenylalanyl-adenylate; PheOH-AMP, adenylate of L-(−)-3-phenyllactic acid; PheH-AMP, adenylate of hydrocinnamic acid.

10.1021/bi701456c CCC: $40.75
Published on Web 12/08/2007

provides an explanation for the surprising adaptability of the genetic code to novel amino acids" (*8*).

To test the hypothesis that the use of acyl-adenylates can overcome the natural selectivity of aaRS, we studied $tRNA^{Phe}$ acylation by acid and α-hydroxy acid analogues of Phe. The primary α-amino group of Phe is crucial for amino acid recognition by phenylalanyl-tRNA synthetase (*20*). At the same time tRNAs acylated with α-hydroxy analogue of Phe can be utilized by a ribosome with formation of proteins containing ester bonds instead of peptide bonds (*21, 22*). This backbone modification proved to be useful as a tool to study ribosome function (*23*) and site directed mutagenesis of protein backbone (*24*).

MATERIALS AND METHODS

Crystalline adenosine 5′-monophosphate (AMP) free acid, L-phenylalanine (1), and *N*,*N*′-dicyclohexylcarbodiimide (DCC) were obtained from the Sigma Chemical Co.; hydrocinnamic acid (Phe-H) was obtained from TCI America; L-(−)-3-phenyllactic acid (Phe-OH) was obtained from Fluka.

Dual-specific Ala/PheYFA2 tRNA was *in vitro* transcribed and gel purified as described elsewhere (*25*). His-tagged yeast phenylalanyl-tRNA synthetase was expressed in *Escherichia coli* from the vector kindly provided by the D. Tirrell lab (Caltech) and purified by Ni-column, followed by dialysis and MonoQ ion exchange chromatography. Kinetic data was analyzed using Kaleidagraph 4.0 (Abelbeck Software).

Synthesis of L-Phenylalanyl Adenylate. The synthesis was made according to the method by Berg (*26*) with several modifications. L-Phenylalanine (2.0 mmol) and AMP (1.92 mmol) were mixed with 3.2 mL of cold water and 10.4 mL of pyridine in a glass-stoppered flask. 8 N HCl (0.25 mL) was added, and the mixture was stirred in an ice bath. DCC (50 mmol), dissolved in 12 mL of pyridine, was added, and the mixture was stirred for about 4 h. The reaction was terminated by the addition of about 150 mL of cold acetone. After 1 min the precipitate of crude L-phenylalanyl adenylate was filtered rapidly with the aid of suction, washed three times with small volumes of cold acetone−ethyl alcohol (60:40) mixture, then with ethyl ether, and air-dried on the filter under suction. The crude product was suspended in 10 mL of ice-cold water and rapidly filtered, followed by three washes with 5 mL of ice-cold water. The washes and original filtrate were combined, pH was adjusted to 3 with HCl, and 100 mL of cold ethanol was added. After overnight precipitation, the precipitate was separated by centrifugation, washed with ethanol, and dried in a SpeedVac evaporator.

The L-phenylalanyl adenylate (Phe-AMP) was isolated by RP-HPLC (ZORBAX SB-C18, 9.4 mm × 25 cm P.N. column) using the linear gradient 0−30% of buffer B (buffer B, CH_3CN; buffer A, 50 mM ammonium acetate pH 4.5), 0.85%/min at a flow-rate 1.5 mL/min. Under these conditions, L-phenylalanyl-adenylate was eluted at 29.3 min (MS (ES) *m*/*z* 495.1 (M + H), 512.2 (M + H_2O), 492.2 (M − H)). Fractions were collected and adenylate was concentrated by lyophilization to almost dryness. The resulting solution was stored in aliquots at −80 °C. Before reaction, adenylates were diluted by 50 mM ammonium acetate buffer pH 4.5 to a desired concentration.

Adenylate of hydrocinnamic acid (PheH-AMP) (m/z 480.1 (M + H), 478.2 (M − H)) and adenylate of L-(−)-3-phenyllactic acid (PheOH-AMP) (*m*/*z* 496.1 (M + 1), 494.2 (M − H)) were synthesized and purified under the same conditions.

Concentration of adenylates was determined spectrophotometerically using the adenosine absorption coefficient. Concentration was corrected by the fraction of adenylate in the preparation, which was determined by HPLC analysis.

Aminoacylation Reaction. The reaction of tRNA aminoacylation using 3′-^{32}P-labeled $tRNA^{Phe}$ was performed as described previously (*27*). A typical reaction mixture (10 μL) contained 50 mM HEPES pH 7.5, 30 mM KCl, 15 mM $MgCl_2$, 3 mM ATP, 0.5 mM DTT, about 0.01 μCi of 3′-[^{32}P]$tRNA^{Phe}$ and nonlabeled tRNA to final concentration 1 μM, 50−300 μM of substrate (appropriate adenylate), 10 U/mL of inorganic pyrophosphatase and yeast phenylalanyl-tRNA synthetases (yPheRS). Aminoacylation at pH 6.0 was performed in the same buffer of the same composition except containing 50 mM MES instead of HEPES. In reaction with adenylate analogues, the concentration of yPheRS was within 30 nM to 500 nM. Reaction mixtures were incubated at room temperature. One microliter samples were withdrawn at the desired times and quenched into 4 μL of 200 mM sodium acetate, pH 5.0, containing 1 unit/μL of nuclease P1 (Sigma) at 0 °C. After 20 min incubation at room temperature, 1 μL of each P1 digestion reaction was spotted on F-cellulose plates (Selector Scientific). ^{32}P-labeled ATP and acylated-AMP were separated by TLC in glacial acetic acid/*n*-butanol/H_2O (5/2/3). The radioactivity was analyzed using Phosphor Imager (Molecular Dynamics).

Hydrolysis Rate of Adenylate Analogues Was Analyzed Using HPLC. Pure adenylate analogues, about 1 OD of each, were incubated at 37 °C in the mixture (1000 μL), which contained 50 mM HEPES pH 7.5, 30 mM KCl, 15 mM $MgCl_2$, and 0.5 mM DTT. 100 μL samples were taken at the desired time and quenched by the adding of acetic acid to pH 4.5 and frozen at −80 °C. Then samples were analyzed on RP-HPLC (Hypes ODS, 5 μm, 4.0 × 20 mm, Agilent column) using the linear gradient 0−30% of buffer B (buffer B, CH_3CN; buffer A, 50 mM ammonium acetate pH 4.5) 1.2%/min at a flow rate 1.5 mL/min. The hydrolysis rate was calculated from the ratio of acyladenylate to AMP at every given time point.

RESULTS

Synthesis of Phenylalanyl-adenylate and the Analogues. The phenylalanyl-adenylate (Phe-AMP) and its acid (PheH-AMP) and α-hydroxy (PheOH-AMP) analogues (Figure 1) were synthesized using the Berg procedure (*26*) followed by HPLC purification of the compounds. The resulting material contained 10 to 30% of AMP as an impurity. This impurity originates from the uncontrolled hydrolysis of adenylates during the purification process and storage. Adenylates are prone to hydrolysis at the neutral pH (Figure 2A) with corresponding half-lives of 0.5, 3 and 32 min for Phe-AMP, PheOH-AMP and PheH-AMP, respectively. The hydrolysis rate was measured at pH 7.5 and 37 °C under the standard reaction conditions for tRNA aminoacylation. The presence of a nucleophilic group in the vicinity of the phosphoester bond apparently catalyzes the hydrolysis. All three adenylates display much higher stability at lower pH (Figure 2B). The half-life of Phe-AMP under these conditions

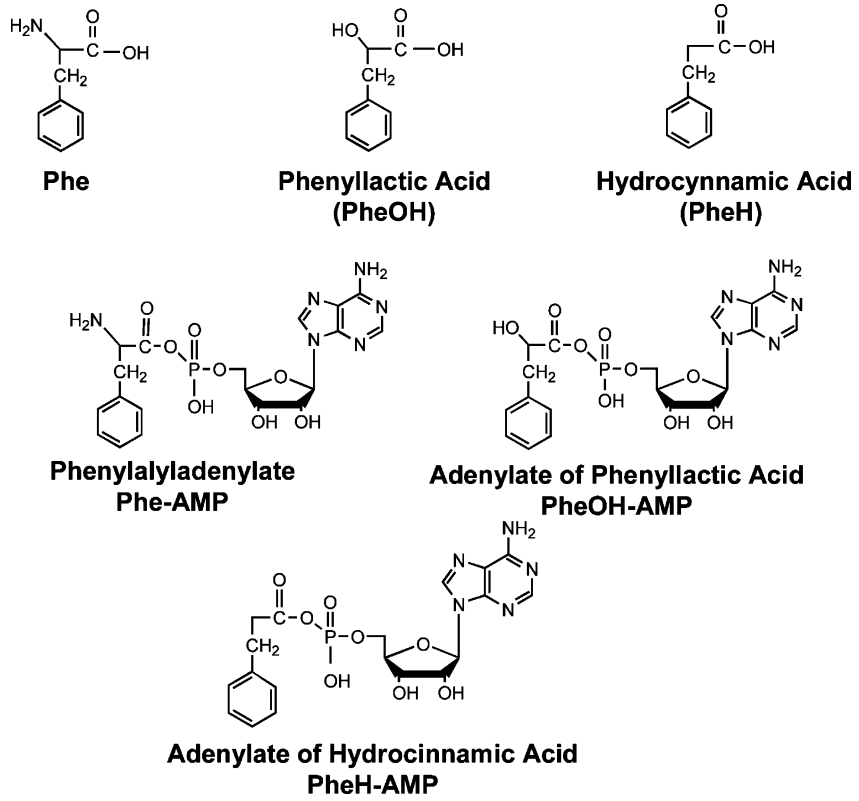

FIGURE 1: Structures of the Phe and phenylalanyladenylate analogues used for tRNA acylation by yeast PheRS. Phenyllactic acid is abbreviated as PheOH, hydrocynnamic acid as PheH, and the corresponding adenylates are designated as Phe-AMP, PheOH-AMP and PheH-AMP, respectively.

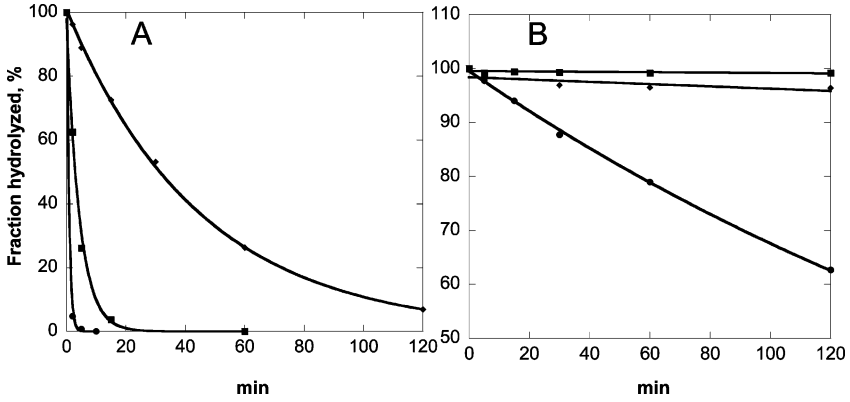

FIGURE 2: Spontaneous hydrolysis of adenylates at pH 7.5 (A) and pH 6 (B). Phe-AMP (●), PheOH-AMP (■),PheH-AMP (◆). Hydrolysis was followed by HPLC analysis of the adenylates solutions incubated for the indicated periods of time.

is 3 h, with the estimated half-lives of other adenylates exceeding 10 h.

tRNA Acylation by Adenylates. Acylation of tRNA was followed by formation of acylated AMP detected by TLC separation of the products of P1 digestion of 3′-^{32}P labeled tRNA (*27*). Acylation of tRNA can be observed upon addition of all three adenylates into the reaction mixture (Figure 3). The acylation products with Phe, PheOH and PheH can be distinctly resolved. The levels of tRNA aminoacylation observed upon addition of Phe-AMP are equal to levels achieved in the complete aminoacylation reaction containing ATP and Phe. The acylation levels achieved in reaction with PheH-AMP and PheOH-AMP analogues are lower. Increase in the reaction time did not result in improvement of acylation by PheOH-AMP because of the rapid adenylate hydrolysis in the reaction mixture (Figure 2), while slightly higher levels of tRNA acylation by PheH-AMP were achieved during longer reaction times. No detectable direct tRNA acylation by PheH and PheOH was observed in complete reactions containing amino acid analogues and ATP (data not shown).

Kinetics of tRNA Acylation by Adenylates. Increase in adenylate concentration did not result in significant increase of the observed levels of tRNA acylation. Moreover, a substrate inhibition is observed at the high concentration of adenylates (data not shown). The most likely reason for the inhibition is the competition with AMP which is present in adenylate preparations or is rapidly generated in the reaction mixture.

Increase in enzyme concentration results in increased levels of tRNA aminoacylation (Figure 4). Nevertheless, the maximal levels of tRNA acylation by PheH-AMP and PheOH-AMP were never higher than 30% of levels observed in reaction with Phe-AMP. The significantly lower levels

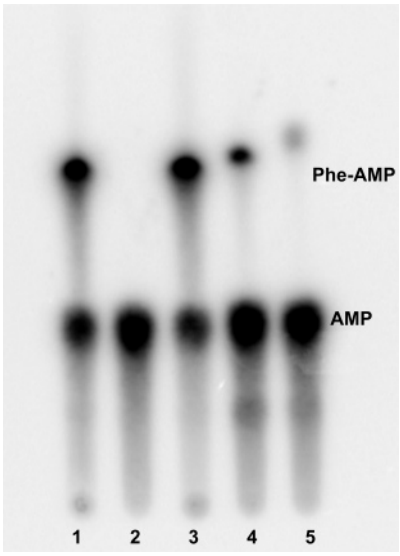

FIGURE 3: TLC analysis of tRNA acylation by phenylalanyl-adenylate and its analogues. Positions of AMP and Phe-AMP are indicated. Reaction was performed at 1 μM tRNA, 500 nM PheRS and 50 μM ATP with 1 mM Phe (line 1); no substrates (line 2), 50 μM of Phe-AMP (line 3), PheOH-AMP (line 4), and PheH (line 5). Reaction time was 5 min for lines 1 and 3 and 4 h for lines 2, 4 and 5.

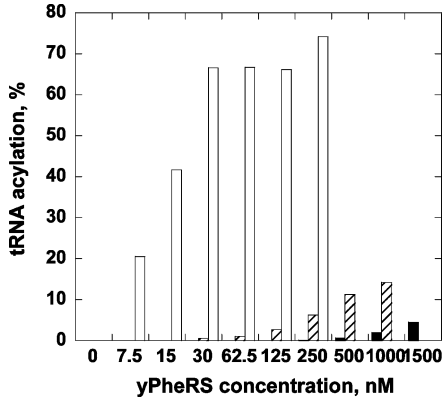

FIGURE 4: tRNA acylation level dependence on the enzyme concentration. 50 μM Phe-AMP (open bars), PheOH-AMP (hatched bars) and PheH-AMP (black bars) were reacted with 1 μM tRNA and indicated concentrations of yPheRS. Incubation time was 5 min for Phe-AMP and PheOH-AMP and 60 min for PheH-AMP. Reaction was performed at pH 6.0. Values presented are the average of three independent measurements with deviation not exceeding 20%.

of acylation by analogues can be explained in the terms of the "plateau" theory (*28*). Since the reaction product (acylated tRNA) is prone to hydrolysis, the observed level of aminoacylation may be the result of the equilibrium between forward acylation rate and deacylation rates.

Rapid nonenzymatic hydrolysis of adenylates makes it technically very difficult to accurately measure the kinetics of tRNA acylation and evaluate the effect of substitution and removal of the Phe primary α-amino group on aminoacylation reaction. Since adenylates demonstrate much higher stability at lower pH, we have measured the kinetics of tRNA acylation at pH 6.0 (Figure 5). Under these conditions the overall reaction rate was significantly slower than at neutral pH, but it became possible to compare the acylation rates between different adenylates.

The substitution of the primary α-amino group of Phe with hydroxyl resulted in a 300-fold decrease in k_{cat}/K_M value, while complete removal of the functional group resulted in a 4000-fold decrease in the value (Table 1). Quite remarkably, the major reduction in catalytic efficiency of aminoacylation came from the decrease in k_{cat} and not from a K_M value. Considering the significantly slower acylation rate displayed by PheH and PheOH adenylate analogues under the reaction conditions, it is possible to suggest that the observed K_M value reflects the actual affinity of adenylates to enzyme. The K_M value of phenylalanyl-adenylate observed in our experiments (1.5 μM) is significantly higher than the previously observed K_D value of 4.4 nM (*29*). This discrepancy can be attributed to the different reaction conditions (pH 6.0 in our experiments and 7.5 in other work) as well as to the unknown ratio between k_2 (rate of amino acid transfer to tRNA) and k_{-1} (rate of adenylate dissociation from the active site) in our experimental conditions.

DISCUSSION

No tRNA acylation was observed when PheOH and PheH were substituting cognate Phe in the aminoacylation reaction. Coupling the same molecules with AMP to form acyl-adenylates resulted in the reactive intermediate, and tRNA acylation was observed. The presence of an AMP moiety provided adenylates with the ability to overcome the PheRS specificity barrier and transfer otherwise inactive amino acid analogues onto tRNA. The substitution of the amino group by hydroxyl resulted in a 300-fold decrease in catalytic efficiency, followed by another 10-fold reduction upon completely removing the functional group (Table 1). The observed loss of transfer efficiency can be attributed to the disruption of a hydrogen bond network in the enzyme active site, which may prevent the correct location of adenylate for the attack by 2′OH of A76 ribose of tRNA.

The correct placement of the phenylalanine substrate should be in agreement with an in-line mechanism for its activation, and is essentially driven by a network of aromatic−aromatic interactions (*20*) and the anchoring of its α-amino and COO groups. In the binary *Th. thermophilus* PheRS−Phe (*30*) complex, the amino group of the substrate forms direct hydrogen bonds with ttGlnα-218 and ttGluα-220 (tt- *Thermus thermophilus*). In the PheRS−PheAMP complex, this group interacts with ttGlnα-218 and ttGluα-220 via the well-ordered water molecule S9 and forms additional hydrogen bonds with ttSerα-180 and ttHisα-178 (*20*).

Multiple sequence alignment between PheRSs isolated from different prokaryotic and eukaryotic sources (Figure 6) shows that residues ttGlnα218 and ttGluα220 are invariant for all referenced sequences. For the two residues ttThrα179 and ttSerα180, conservation should be assigned to the hydroxyl group, which occurs in these residues. Amino acids Ser and Thr substitute in yeast PheRS for one another at positions 179 and 180. ttHisα178 in some cases is replaced by glutamine, which is uncharged but has a polar amide group with extensive hydrogen-bonding capacity. Thus, all residues that are involved in binding of the α-amino and COO groups of cognate amino acid in yeast PheRS most probably retain their positions in the active site observed in functional complexes of bacterial enzyme.

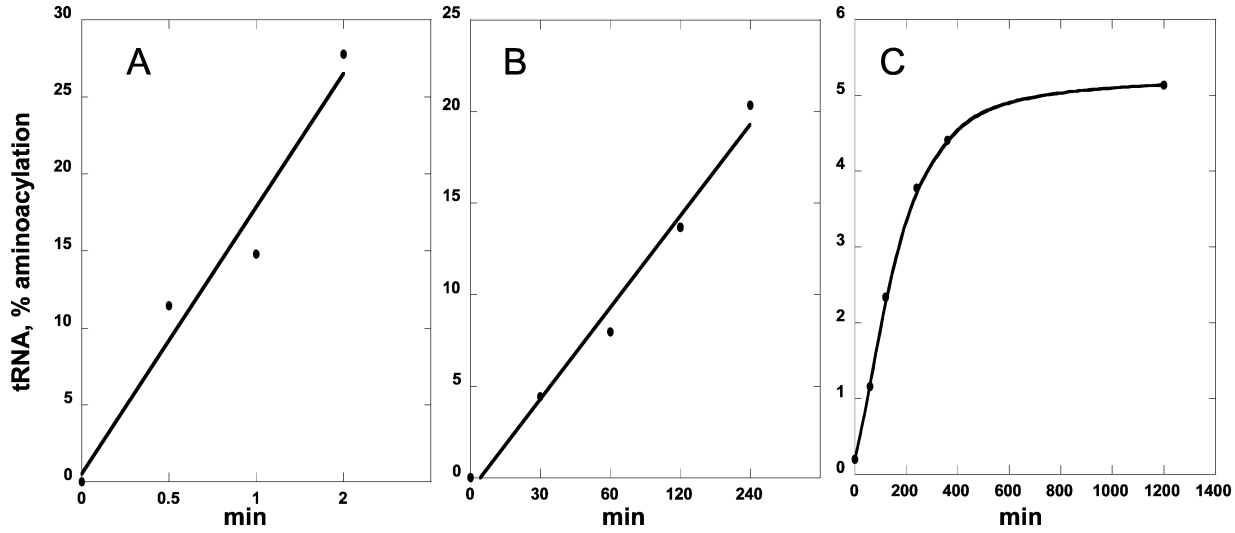

FIGURE 5: Kinetics of tRNA aminoacylation at pH 6.0 by yPheRS: Phe-AMP (A); PheOH (B); PheH (C). Acylation rate was measured at 1 μM tRNA, 50 μM adenylates, 50 nM yPheRS in A and 500 nM in B and C.

Table 1: Kinetic Constants of tRNA Aminoacylation by Adenylates[a]

adenylate	k_{cat}, min^{-1}	K_M, μM	k_{cat}/K_M	relative catalytic efficiency
Phe-AMP	7.1	1.5	4.7	1
PheOH-AMP	0.14	9.3	1.5×10^{-2}	3.2×10^{-3}
PheH-AMP	3.3×10^{-4}	2.8	1.2×10^{-4}	2.5×10^{-5}

[a] The values are the average of at least two independent measurements with deviation within 20% for K_M and 30% for k_{cat}.

The saturation of all hydrogen bonds in the enzyme−substrate complex occurs through the concerted move of the structural elements in the active site and induced-fit adjustment of the substrates to allow the phenylalanylation reaction. In this context, the binding of $PheNH_2$ in the amino acid binding pocket leads to the formation of the network of hydrogen bonds between the α-amino group and yThr331 (ttSer180), yGlu375 (ttGlu220) and yGln373 (ttGln218). When the primary α-amino group of $PheNH_2$ is substituted with hydroxyl, its placement in the active site may induce a shift of the flexible side chain of the yGlu375 and correspondingly cause the formation of an intramolecular hydrogen bond between its carboxylic group and the side chain of yThr331. Then atoms from the second carboxylic group of yGlu375 and from the OE1 atom of yGln373 create a negatively charged environment that induces electrostatic repulsion with like-charged moiety of α-hydroxy amino acid. This will change the orientation of the PheOH, and its carboxylic group will be found in a position not favorable for a nucleophilic attack on the α-phosphate of ATP.

Stacking interactions between yPhe371 and the adenylate ring demonstrate the highest stability among all interactions in which Phe-AMP is involved (*18*). Anchoring of the adenine ring by protein is further reinforced by interaction with the hydrophobic part of class II invariant yArg464 (Figure 7). From the structure of various functional complexes (binary, Phe, AMP, ternary) it follows that it is the formation of the phenylalanyl-adenylate in the course of aminoacylation reaction or the binding of the adenylate analogue which exerts the most pronounced effect on the active site rearrangement. Thus the H-bond between the N6 atoms of adenine and interactions with carboxylates of yGlu361 give rise to the movement of motif 2 and the "helical" loops toward the position of bound PheOH-AMP. This in turn leads to the formation of H-bonds between guanidinium groups of yArg359 with O1P atom of PheOH-AMP. Moreover, the position of the sugar moiety will be additionally stabilized by the interaction of its 3′OH group with carbonyl oxygen of yIle431. It looks as adenylate binding induces a "quasi-cooperative" effect: the anchoring of the PheOH-AMP adenine ring triggers rearrangement of motif 2 and "helical" loops accompanied by involvement into the binding process of additional residues yGlu361, yArg359, and yIle43, which, in turn, reinforces stability of the binary complex. Such a multistep adjustment, including aromatic−aromatic interaction of the phenyl ring of PheOH-AMP, locks the ligand into a position close to the position of the native intermediate. The proximity of the PheOH-AMP binding mode to the native intermediate makes possible the acylation of tRNA with non-natural amino acids.

Unexpectedly, the major reduction in catalytic efficiency came not from a decrease in K_M but from reduced k_{cat}. The Phe-AMP and both its analogues display similar apparent K_M values. Since the measured acylation rate is relatively slow, one might expect that apparent K_M values are close to K_D of the adenylates, and thus reflect the affinity of the intermediate to the enzyme. Thus, the presence of the primary amino group is important for the adenylate reactivity in the amino acid transfer reaction, with a specificity being defined not by the different affinity of the analogues but by the kinetic discrimination based on the different tRNA transfer rates. The exact role of the correct positioning of the primary α-amino group and carboxyl group of cognate substrates and aminoacyl adenylate intermediates is exemplified by revealing significant conformational changes in the acceptor end of $tRNA^{Phe}$ observed upon formation of the enzyme's ternary complex. In the presence of a nonhydrolyzable phenylalanyl-adenylate analogue, where the carbonyl group is substituted by the methylene group, the single-stranded 3′ end of $tRNA^{Phe}$ exhibits a hairpin conformation that leads to a nonproductive binding mode of reactants (*18*). The unfavorable position of the terminal ribose stems from the absence of the α-carbonyl oxygen in the analogue. Based on structural data, we may speculate that the transition from a fully

```
TTH     168  EVEGRLLLRTHTSPMQVRYMVA---HT--PPFRIVVPGRVFRFEQTDATHEAVFHQLEGL
BAC     153  ---EETLMRTQTSPVQTRTMEK---HEGKGPVKIICPGKVYRRDNDDATHSHQFMQIEGL
ECO     153  ---TTRLLRTQTSGVQIRTMKA---QQ--PPIRIIAPGRVYRND-YDQTHTPMFHQMEGL
HEL     154  ---DHKLLRTHTSPVQIHTMQ-----EQTPPIKMICLGETFRRD-YDLTHTPMFHQIEGL
Human   317  DEARKNLLRTHTTSASARALYRLAQKKPFTPVKYFSIDRVFRNETLDATHLAEFHQIEGV
CAE     317  EEAQKNVLRTHTTAVSARQLYQLAQ-EGFRPSKLFSIDRVFRNETLDATHLAEFHQVEGV
YST     319  EECQKLVLRTHSTAISARMLHDLAKD-P-KPTRLFSIDRVFRNEAVDATHLAEFHQVEGV
MET     322  NVSRRLILRTHTTASSIRYLASLSDEEKNKPHKVFCIDRVFRNEAIDYKHLPEFYQCEGI
Cons             ::**:::  . : :          * : .  ...:* :  * .*   * * **:

TTH     223  VVGE--GIAMAHLKGAIYELAQALFGPDSKVRFQPVYFPFVEPGAQFAV-----------
BAC     216  VVDK--NISMSDLKGTLELVAKKMFGQDREIRLRPSFFPFTEPSVEVDVTCFKCGGNGCS
ECO     213  IVDT--NISFTNLKGTLHDFLRNFFEEDLQIRFRPSYFPFTEPSAEVDV-----------
HEL     205  VVDQKGNIRFTHLKGVIEDFLHYFFG-GVKLRWRSSFFPFTEPSAEVDISCVFCKQEGCR
HUM     377  VADH--GLTLGHLMGVLREFFTKL-G-ITQLRFKPAYNPYTEPSMEVFS-----------
CAE     376  IAEK--NLSLAHLIGIFTEFFKKL-G-ITNLRFKPTYNPYTEPSMEIFA-----------
YST     378  LADY--NITLGDLIKFMEEFFERM-G-VTGLRFKPTYNPYTEPSMEIFS-----------
MET     382  IMDD--NVNFNNLIGVLKEFLNRL-G-FEKVRFRPAYFPFTEPSLEAEV-----------
Cons         :     .: : .*   :  .   :      :* :. : *:.**. :

TTH     270  WWPEG-GKWLELGGAGMVHPKVFQAVDAYRERLGLPPAYRGVTGFAFGLGVERLAMLRYG
BAC     274  VC-KG-TGWIEILGAGMVHPNVLKMA-------GFDP--KEYQGFAFGMGVERIAMLKYG
ECO     260  MG-KN-GKWLEVLGCGMVHPNVLRNV-------GIDP--EVYSGFAFGMGMERLTMLRYG
HEL     263  V-CSH-TGWLEVLGCGMVNNAVFEAI-------GY----ENVSGFAFGMGIERLAMLTCQ
HUM     422  YH-QGLKKWVEVGNSGVFRPEMLLPM-------GLP---ENVSVIAWGLSLERPTMIKYG
CAE     421  YH-QGLTKWVEIGNSGMFRPEMLLPM-------GLP---ADVNVAGYGLSLERPTMIKYG
YST     423  WH-EGLQKWVEIGNSGMFRPEMLESM-------GLP---KDLRVLGWGLSLERPTMIKYK
MET     427  YL-EG-KGWLEILGAGIFRPEVLEPI-------GIEK-----PVLAWGIGFSRLAMLRYG
Cons            .    *:*: ..*:..  ::          *           .:*:...* :*:
```

FIGURE 6: Multiple sequence alignment of α-subunit PheRS isolated from different sources. The origins are *Homo sapiens*, HUM; *Thermus thermophilus*, TTH; *Bacillus subtilis*, BAC; *Helicobacter pillory*, HEL; *Methanococcus jannaschii*, MET; *Caenorhabditis elegans*, CAE; *Escherichia coli*, ECO; *Saccharomyces serevisiae*, YST. The alignment was carried out using the program CLUSTAL W (1.83).

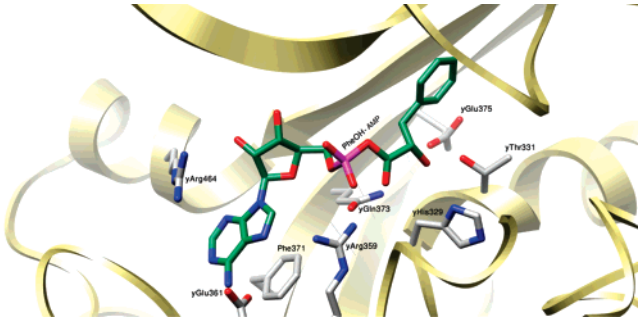

FIGURE 7: Model of PheOH-AMP in the synthetic active site of yPheRS. The amino acid residues presented in the picture for the most part are PheRSor class II invariants. Conformation of PheOH-AMP ligand and backbone of the polypeptide chain are taken from ternary complex structure of *Th. thermophilus* PheRS (*18*).

productive to a partially productive or nonproductive binding mode associated with the alterations of small substrates most probably will result in conformational changes of the of CCA end. In spite of all this, the majority of intermolecular PheRS−tRNA contacts will not change. This may account for the reduction of the catalytic coefficient k_{cat}/K_M when one reduces $k_{cat.}$ Further kinetic analysis is required to delineate the exact role of the amino group in the reaction mechanism.

In this work we have demonstrated the incorporation of acid and α-hydroxy acid analogues of the amino acid into tRNA. The α-hydroxyacyl-tRNAs can be utilized in the formation of proteins with ester bonds (*21*, *22*). The site-specific incorporation of ester bonds into proteins can be used to evaluate the functional role of backbone interactions in maintaining protein structure and function (*11*, *24*) and ribosome function (*23*). Currently, the, incorporation of α-hydroxy acids into proteins can be achieved either by chemical synthesis of acyl-tRNA (*13*) or by aminoacyl-tRNA oxidation by nitric acid (*23*). While the synthetic procedure is laborious, the latter procedure results in a variety of oxidation products. The procedure suggested in this paper provides a simpler and cleaner way of making α-hydroxyacyl-tRNAs using α-hydroxyacyl-adenylate as a substrate for the enzyme.

Our results are the first demonstration that the use of synthetic aminoacyl-adenylates as substrates in tRNA aminoacylation reaction may provide a way for incorporation of unnatural amino acids into tRNA and consequently into proteins. The specificity of aaRS toward adenylates seems to be lower than toward amino acids. Using adenylate analogues we were able to enzymatically acylate tRNA with amino acid analogues which were otherwise completely inactive in direct aminoacylation reaction, thus bypassing the natural mechanisms ensuring the selectivity of tRNA aminoacylation. The advantage of this approach is that it exploits the natural promiscuity of aaRSs and does not require any manipulations with existing enzymes, while the synthesis of the aminoacyl-adenylates is relatively simple and straightforward. Using synthetic aminoacyl-adenylates may become a useful tool for unnatural amino acid incorporation for *in vitro* protein translation applications.

ACKNOWLEDGMENT

We are grateful to Dr. M. Yarus for helpful discussion.

REFERENCES

1. Hendrickson, T. L., de Crecy-Lagard, V., and Schimmel, P. (2004) Incorporation of nonnatural amino acids into proteins, *Annu. Rev. Biochem. 73*, 147−176.

2. Ibba, M., and Soll, D. (1999) Quality control mechanisms during translation, *Science 286*, 1893−1897.
3. Ibba, M., and Soll, D. (2000) Aminoacyl-tRNA synthesis, *Annu. Rev. Biochem. 69*, 617−650.
4. Datta, D., Wang, P., Carrico, I. S., Mayo, S. L., and Tirrell, D. A. (2002) A designed phenylalanyl-tRNA synthetase variant allows efficient in vivo incorporation of aryl ketone functionality into proteins, *J. Am. Chem. Soc. 124*, 5652−5653.
5. Kiick, K. L., Saxon, E., Tirrell, D. A., and Bertozzi, C. R. (2002) Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation, *Proc. Natl. Acad. Sci. U.S.A. 99*, 19−24.
6. Sharma, N., Furter, R., Kast, P., and Tirrell, D. A. (2000) Efficient introduction of aryl bromide functionality into proteins in vivo, *FEBS Lett. 467*, 37−40.
7. Tang, Y., and Tirrell, D. A. (2002) Attenuation of the editing activity of the Escherichia coli leucyl-tRNA synthetase allows incorporation of novel amino acids into proteins in vivo, *Biochemistry 41*, 10635−10645.
8. Turner, J. M., Graziano, J., Spraggon, G., and Schultz, P. G. (2006) Structural plasticity of an aminoacyl-tRNA synthetase active site, *Proc. Natl. Acad. Sci. U.S.A. 103*, 6483−6488.
9. Xie, J., and Schultz, P. G. (2005) Adding amino acids to the genetic repertoire, *Curr. Opin. Chem. Biol. 9*, 548−554.
10. Santoro, S. W., Anderson, J. C., Lakshman, V., and Schultz, P. G. (2003) An archaebacteria-derived glutamyl-tRNA synthetase and tRNA pair for unnatural amino acid mutagenesis of proteins in Escherichia coli, *Nucleic Acids Res. 31*, 6700−6709.
11. Eisenhauer, B. M., and Hecht, S. M. (2002) Site-specific incorporation of (aminooxy)acetic acid into proteins, *Biochemistry 41*, 11472−11478.
12. Klarmann, G. J., Eisenhauer, B. M., Zhang, Y., Sitaraman, K., Chatterjee, D. K., Hecht, S. M., and Le, Grice, S. F. (2004) Site- and subunit-specific incorporation of unnatural amino acids into HIV-1 reverse transcriptase, *Protein Expression Purif. 38*, 37−44.
13. Heckler, T. G., Chang, L. H., Zama, Y., Naka, T., Chorghade, M. S., and Hecht, S. M. (1984) T4 RNA ligase mediated preparation of novel "chemically misacylated" tRNAPheS, *Biochemistry 23*, 1468−1473.
14. Kourouklis, D., Murakami, H., and Suga, H. (2005) Programmable ribozymes for mischarging tRNA with nonnatural amino acids and their applications to translation, *Methods 36*, 239−244.
15. Murakami, H., Saito, H., and Suga, H. (2003) A versatile tRNA aminoacylation catalyst based on RNA, *Chem. Biol. 10*, 655−662.
16. Belrhali, H., Yaremchuk, A., Tukalo, M., Larsen, K., Berthet-Colominas, C., Leberman, R., Beijer, B., Sproat, B., Als-Nielsen, J., Grubel, G., and et al. (1994) Crystal structures at 2.5 angstrom resolution of seryl-tRNA synthetase complexed with two analogs of seryl adenylate, *Science 263*, 1432−1436.
17. Forrest, A. K., Jarvest, R. L., Mensah, L. M., O'Hanlon, P. J., Pope, A. J., and Sheppard, R. J. (2000) Aminoalkyl adenylate and aminoacyl sulfamate intermediate analogues differing greatly in affinity for their cognate Staphylococcus aureus aminoacyl tRNA synthetases, *Bioorg. Med. Chem. Lett. 10*, 1871−1874.
18. Moor, N., Kotik-Kogan, O., Tworowski, D., Sukhanova, M., and Safro, M. (2006) The crystal structure of the ternary complex of phenylalanyl-tRNA synthetase with tRNAPhe and a phenylalanyl-adenylate analogue reveals a conformational switch of the CCA end, *Biochemistry 45*, 10572−10583.
19. Kotik-Kogan, O., Moor, N., Tworowski, D., and Safro, M. (2005) Structural basis for discrimination of L-phenylalanine from L-tyrosine by phenylalanyl-tRNA synthetase, *Structure 13*, 1799−1807.
20. Fishman, R., Ankilova, V., Moor, N., and Safro, M. (2001) Structure at 2.6 A resolution of phenylalanyl-tRNA synthetase complexed with phenylalanyl-adenylate in the presence of manganese, *Acta Crystallogr., Sect. D: Biol. Crystallogr. 57*, 1534−1544.
21. Fahnestock, S., and Rich, A. (1971) Ribosome-catalyzed polyester formation, *Science 173*, 340−343.
22. Fahnestock, S., and Rich, A. (1971) Synthesis by ribosomes of viral coat protein containing ester linkages, *Nat. New Biol. 229*, 8−10.
23. Bieling, P., Beringer, M., Adio, S., and Rodnina, M. V. (2006) Peptide bond formation does not involve acid-base catalysis by ribosomal residues, *Nat. Struct. Mol. Biol. 13*, 423−428.
24. England, P. M., Zhang, Y., Dougherty, D. A., and Lester, H. A. (1999) Backbone mutations in transmembrane domains of a ligand-gated ion channel: implications for the mechanism of gating, *Cell 96*, 89−98.
25. Pleiss, J. A., Wolfson, A. D., and Uhlenbeck, O. C. (2000) Mapping contacts between Escherichia coli alanyl tRNA synthetase and 2′ hydroxyls using a complete tRNA molecule, *Biochemistry 39*, 8250−8258.
26. Berg, P. (1958) The chemical synthesis of amino acyladenylates, *J. Biol. Chem. 233*, 608−611.
27. Wolfson, A. D., and Uhlenbeck, O. C. (2002) Modulation of tRNAAla identity by inorganic pyrophosphatase, *Proc. Natl. Acad. Sci. U.S.A. 99*, 5965−5970.
28. Dietrich, A., Kern, D., Bonnet, J., Giege, R., and Ebel, J. P. (1976) Interpretation of tRNA-mischarging kinetics, *Eur. J. Biochem. 70*, 147−158.
29. Baltzinger, M., Lin, S. X., and Remy, P. (1983) Yeast phenylalanyl-tRNA synthetase: symmetric behavior of the enzyme during activation of phenylalanine as shown by a rapid kinetic investigation, *Biochemistry 22*, 675−681.
30. Reshetnikova, L., Khodyreva, S., Lavrik, O., Ankilova, V., Frolow, F., and Safro, M. (1993) Crystals of the phenylalanyl-tRNA synthetase from Thermus thermophilus HB8 complexed with tRNA(Phe), *J. Mol. Biol. 231*, 927−929.

BI701456C