
Functional group recognition at the aminoacylation and editing sites of *E. coli* valyl-tRNA synthetase

KEITH D. TARDIF¹ and JACK HOROWITZ

Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, Iowa 50011, USA

ABSTRACT

To correct misactivation and misacylation errors, *Escherichia coli* valyl-tRNA synthetase (ValRS) catalyzes a tRNA^{Val}-dependent editing reaction at a site distinct from its aminoacylation site. Here we examined the effects of replacing the conserved 3'-adenosine of tRNA^{Val} with nucleoside analogs, to identify structural elements of the 3'-terminal nucleoside necessary for tRNA function at the aminoacylation and editing sites of ValRS. The results show that the exocyclic amino group (N6) is not essential: purine riboside-substituted tRNA^{Val} is active in aminoacylation and in stimulating editing. Presence of an O6 substituent (guanosine, inosine, xanthosine) interferes with aminoacylation as well as posttransfer and total editing (pre- plus posttransfer editing). Because ValRS does not recognize substituents at the 6-position, these results suggest that an unprotonated N1, capable of acting as an H-bond acceptor, is an essential determinant for both the aminoacylation and editing reactions. Substituents at the 2-position of the purine ring, either a 2-amino group (2-aminopurine, 2,6-diaminopurine, guanosine, and 7-deazaguanosine) or a 2-keto group (xanthosine, isoguanosine), strongly inhibit both aminoacylation and editing. Although aminoacylation by ValRS is at the 2'-OH, substitution of the 3'-terminal adenosine of tRNA^{Val} with 3'-deoxyadenosine reduces the efficiency of valine acceptance and of posttransfer editing, demonstrating that the 3'-terminal hydroxyl group contributes to tRNA recognition at both the aminoacylation and editing sites. Our results show a strong correlation between the amino acid accepting activity of tRNA and its ability to stimulate editing, suggesting misacylated tRNA is a transient intermediate in the editing reaction, and editing by ValRS requires a posttransfer step.

Keywords: tRNA; aminoacyl-tRNA synthetase; translation; aminoacylation; nucleoside analogs; translational editing

INTRODUCTION

In a two-step reaction that constitutes the first step of protein biosynthesis, each aminoacyl-tRNA synthetase esterifies a specific amino acid to the ribose of the universally conserved 3'-terminal adenosine of its set of cognate tRNA isoacceptors. The fidelity of this reaction is crucial for the accuracy of protein synthesis. Several aminoacyl-tRNA synthetases, however, have difficulty discriminating between cognate and structurally similar amino acids, resulting in misactivation of noncognate amino acids and misacylation of tRNA. To minimize errors and maintain translational fidelity, these enzymes catalyze a tRNA-dependent (Fersht and Kaethner 1976; Jakubowski and Fersht 1981) proof-

reading (editing) reaction that hydrolyzes either the incorrectly activated aminoacyl-adenylate (pretransfer editing) or the mischarged tRNA (posttransfer editing) (for review, see Jakubowski and Goldman 1992). Studies with the class Ia synthetases IleRS (Schmidt and Schimmel 1994; Lin et al. 1996), valyl-tRNA synthetase (ValRS; Lin and Schimmel 1996; Lin et al. 1996), and LeuRS (Chen et al. 2000; Cusack et al. 2000; Mursinna et al. 2001) showed that the aminoacylation and editing active sites of these enzymes are spatially separate; an ~200-amino-acid insert in the aminoacylation site, designated CP1, is responsible for the editing activity (Schmidt and Schimmel 1995; Lin et al. 1996; Nureki et al. 1998; Fukai et al. 2000, 2003; Lincecum et al. 2003). Cognate tRNAs specifically trigger transfer of misactivated amino acids from the synthetic active site to the editing active site, accounting for the tRNA dependence of the editing reaction (Silvian et al. 1999; Fukai et al. 2000; Bishop et al. 2002, 2003).

Chemical and enzymatic modification studies demonstrated the importance of the 3'-end of tRNA in the aminoacylation reaction (for review, see Sprinzl and Cramer 1979) and in translational editing (Tardif et al. 2001; Tardif

Reprint requests to: Jack Horowitz, Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA 50011, USA; e-mail: jhoro@iastate.edu; fax: (515) 294-0453.

¹**Present address:** Department of Microbiology, Program of Molecular Biology, University of Colorado Health Sciences Center, Denver, CO 80262, USA.

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and Horowitz 2002). Altering the 3'-terminal adenosine (Best and Novelli 1971; Tal et al. 1972; Rether et al. 1974; Von der Haar and Gaertner 1975) or its ribose moiety (Uziel and Jacobson 1974) results in complete loss or considerable decrease in aminoacylation efficiency, largely due to a decrease in k_{cat} . 3'-end-modified tRNAs are also defective in editing (Tardif et al. 2001) and are readily misacylated (Tamura et al. 1994; Tardif et al. 2001; Nordin and Schimmel 2002).

Although these results imply that the universally conserved 3'-terminal adenosine is specifically recognized at both the aminoacylation and the editing sites of synthetases, recent experiments have shown that *Escherichia coli* tRNA^{Val} (Liu and Horowitz 1994; Tamura et al. 1994; Tardif et al. 2001) and several other tRNAs (Liu et al. 1998) with base substitutions at the 3'-end are readily aminoacylated. 3'-end-modified tRNA^{Val}, however, does not stimulate the editing activity of ValRS, nor can the modified tRNA^{Val}, misacylated with threonine, be edited (Tardif et al. 2001), suggesting that the aminoacylation active site of the enzyme recognizes different features of the 3'-terminal nucleotide than the editing active site.

In this study we examined the role of functional groups on the 3'-terminal nucleoside of *E. coli* tRNA^{Val} in specific recognition of the 3'-end of tRNA^{Val} by ValRS. By replacing the universally conserved A76 with nucleoside analogs having different chemical substituents on the base or sugar, we probed the contribution of individual substituents to the specificity of recognition at the aminoacylation and editing active sites of the enzyme, and we compared the structural elements essential for function at each site.

RESULTS

Functional groups of the 3'-terminal base of tRNA^{Val} recognized at the aminoacylation and editing sites of valyl-tRNA synthetase

The universally conserved 3'-adenosine of *E. coli* tRNA^{Val} was replaced with nucleotide analogs by using T4 RNA ligase to add bisphosphate derivatives of the analogs to tRNA^{Val} transcripts lacking the terminal adenosine. After dephosphorylation with calf intestinal phosphatase, the product of a control ligation with pAp was fully active in both aminoacylation and editing (Tables 1, 3). Base analogs substituted for the 3'-terminal adenine differ from adenine either in the heterocyclic ring or in exocyclic substituents (see structures in Fig. 1) and thus have different reactivities and H-bonding characteristics. By determining the effect of substitutions on the ability of the modified tRNAs to accept valine and to stimulate translational editing, we were able to examine and compare the specific recognition of individual

TABLE 1. Valine acceptance by tRNA^{Val} with base analog substitutions at the 3'-end

3'-Analog substitution	Steady-state level of aminoacylation (pmole/A ₂₆₀)	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)	Relative aminoacylation efficiency
Adenosine	1285	3.15	(1.0)
3-Deazaadenosine	1471	2.01	0.64
7-Deazaadenosine	1551	1.65	0.52
Purine riboside	1428	2.27	0.72
Inosine	1122	0.35	0.11
2-Aminopurine	945	0.27	0.09
2,6-Diaminopurine	1284	0.16	0.05
Xanthosine	1337	0.04	0.01
Isoguanosine	1022	0.55	0.18
7-Deazaguanosine	849	0.07	0.02
Guanosine	1120	0.02	0.01

structural elements of the 3'-terminal nucleoside at the aminoacylation and the editing sites of ValRS.

Recognition of functional groups at the aminoacylation site of ValRS

The importance of the heterocyclic ring nitrogens of the 3'-terminal adenosine of tRNA^{Val} for aminoacylation by ValRS was tested by inserting 7-deazaadenosine (7-deazaA) and 3-deazaadenosine (3-deazaA), which have, respectively, the N7 and N3 ring nitrogens replaced by carbon, at the 3'-end of tRNA^{Val}. *E. coli* tRNA^{Val} terminating in 7-deazaA is a good substrate for ValRS: it is fully aminoacylated with valine with an efficiency (k_{cat}/K_m) half that of wild-type tRNA^{Val}(A76) (Table 1). Similarly, tRNA^{Val} with 3'-terminal 3-deazaA is completely charged with valine, with aminoacylation efficiency 64 percent that of tRNA^{Val}(A76) (Table 1). Evidently the N7 and N3 ring nitrogens of the 3'-terminal adenosine are not essential for effective tRNA recognition at the aminoacylation site of ValRS.

The exocyclic 6-amino group of adenosine (N6) is also not a positive determinant for aminoacylation of tRNA^{Val} by ValRS. *E. coli* tRNA^{Val} with purine riboside, which lacks the exocyclic 6-amino group of adenosine (Fig. 1) in place of the normal 3'-terminal adenosine is fully charged by ValRS (Table 1). It is 72% as efficient as the wild-type tRNA in accepting valine (Table 1), with little change in k_{cat} and only a twofold increase in K_m (Table 2).

Replacing the 3'-A76 with inosine decreases aminoacylation efficiency more than ninefold compared to wild-type tRNA^{Val} (Table 1), due largely to a 13-fold decrease in k_{cat} (Table 2); 3'-inosine-substituted tRNA^{Val} can, however, be fully charged with valine (Table 1). Inosine differs from adenosine at both the 1- and 6-positions of the purine ring; it has a 6-carbonyl (O6) substituent in place of the 6-NH₂ group of adenosine, and its N1-nitrogen is protonated (Fig. 1). These chemical differences alter the H-bonding proper-

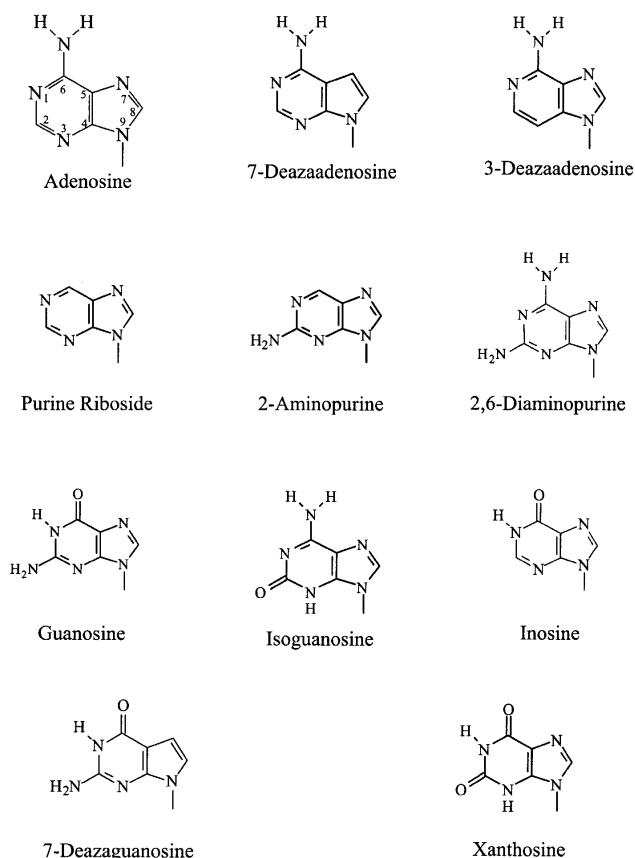


FIGURE 1. Chemical structures of nucleobase analogs inserted at the 3'-end of tRNA^{Val} replacing the normal A76.

ties of the base, and either or both may account for the observed inhibition of aminoacylation (see Discussion).

A 2-amino substituent (N2) on the 3'-terminal base greatly reduces the efficiency of aminoacylation of tRNA^{Val}. Substituting A76 of *E. coli* tRNA^{Val} with 2,6-diaminopurine, which differs from adenosine in having an additional exocyclic amino group at the 2-position of the purine ring (Fig. 1), lowers the efficiency of valine acceptance 95% (Table 1); however, this tRNA^{Val} variant can be fully aminoacylated with valine (Table 1). The adverse effect of an exocyclic 2-amino group on aminoacylation is also evident when 2-aminopurine, an analog of adenine in which the amino group on the purine ring is shifted from the 6- to the 2-position (Fig. 1), replaces A76 of tRNA^{Val}. The efficiency of valine charging is reduced eightfold compared to that of the purine nucleoside-substituted tRNA (and 11-fold relative to wild-type tRNA^{Val}; Table 1), largely the result of a 20-fold decrease in k_{cat} (Table 2).

To test the combined effect of both an exocyclic 6-keto group (with a protonated N1-nitrogen) and a 2-NH₂ substituent, A76 was replaced with G76. Although tRNA^{Val} terminating in 3'-guanosine is completely charged with valine, it is a very poor substrate for ValRS; the efficiency of valine acceptance is two orders of magnitude lower than

that of wild-type tRNA^{Val}(A76) (Table 1; also see Liu and Horowitz 1994; Tamura et al. 1994; Tardif et al. 2001), primarily the result of a 300-fold decrease in k_{cat} (Liu and Horowitz 1994). Presence of the 2-NH₂ group reduces the efficiency of aminoacylation of tRNA(G76) 11-fold relative to that of tRNA^{Val} terminating in 3'-inosine (Table 1), and the O6 substituent lowers valine charging efficiency by ninefold compared to that of 2-aminopurine-substituted tRNA^{Val} (Table 1).

A keto group at the 2-position (O2) of the purine ring also adversely affects recognition of the 3'-terminal base of tRNA^{Val} at the aminoacylation site of ValRS. This is best exemplified by experiments with *E. coli* tRNA^{Val} terminating with 3'-isoguanosine, which resembles adenosine in having an exocyclic 6-amino group (N6) and an unprotonated N1 ring nitrogen (Fig. 1), but has a carbonyl at the 2-position (O2; see Fig. 1). Although the aminoacylation level of 3'-isoguanosine-substituted tRNA^{Val} reaches 1022 pmole per A₂₆₀ unit (Table 1), it is aminoacylated at only 18% the rate of wild-type tRNA^{Val} (A76) (Table 1), due mainly to a 14-fold decrease in k_{cat} (Table 2).

The inhibitory effect of an O2 substituent on aminoacylation is substantiated by studies with *E. coli* tRNA^{Val} terminating in 3'-xanthosine. Xanthosine, which like inosine has a carbonyl group at position 6 (O6) and a proton on the N1-nitrogen, has an additional keto substituent at the 2-position (O2) of the purine ring (Fig. 1). Although *E. coli* tRNA^{Val} terminating in 3'-xanthosine can be charged to high levels (1337 pmole/A₂₆₀), it is a very poor substrate for aminoacylation by ValRS, having an aminoacylation efficiency an order of magnitude lower than that of tRNA^{Val} with a 3'-terminal inosine (and almost two orders of magnitude lower than that of the wild-type tRNA; Table 1).

Functional group requirements for editing by ValRS

Mutational analysis has shown that replacing the 3'-terminal adenosine of tRNA^{Val} affects aminoacylation and editing differently (Liu and Horowitz 1994; Tamura et al. 1994; Tardif et al. 2001). To compare the functional group requirements at the 3'-end of tRNA^{Val} for aminoacylation by ValRS with those for editing, the effects of analog-substituted tRNA^{Val} on proofreading were examined by following

TABLE 2. Kinetic parameters for valine acceptance by tRNA^{Val} with base analog substitutions at the 3'-end

Analog substitution	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	Relative (k_{cat}/K_m)
Adenosine	4.3	13.90	3.23	(1)
Purine riboside	8.8	14.4	1.63	0.50
2-Aminopurine	5.0	0.68	0.14	0.043
Isoguanosine	2.0	0.99	0.50	0.15
Inosine	4.8	1.06	0.22	0.068

the tRNA-dependent hydrolysis of ATP (in the presence of the noncognate amino acid threonine) that is diagnostic of the overall editing reaction (the sum of pre- and posttransfer editing).

By inserting 3-deazaA and 7-deazaA at the 3'-end of *E. coli* tRNA^{Val} assessed the contribution of the N7 and N3 ring nitrogens to tRNA recognition at the editing site of ValRS. Both the 3'-3-deazaA and the 3'-7-deazaA derivatives of tRNA^{Val} are very active in promoting the editing reaction of ValRS. They are, respectively, 97% and 77% as efficient as wild-type tRNA^{Val} and are, therefore, not readily misacylated with threonine (Table 3). These results indicate that the N7 and N3 ring nitrogens of the 3'-terminal purine are not necessary for productive recognition of tRNA^{Val} at the editing site of ValRS.

3'-Purine riboside-substituted tRNA^{Val} is also active in stimulating editing and is not misacylated with threonine (Table 3). Because purine riboside lacks an exocyclic amino group, the N6 substituent on the 3'-terminal base of *E. coli* tRNA^{Val}, like the N7 and N3 functional groups, is not essential for tRNA recognition at the editing site.

E. coli tRNA^{Val} with a 3'-inosine substitution fails to stimulate the editing activity of ValRS (Table 3), and as a result is misacylated with threonine to a level of 153 pmole/A₂₆₀ (Table 3). Transfer RNAs terminating in guanosine, 7-deazaguanosine, and xanthosine, which, like inosine, have an exocyclic carbonyl group at the 6-position (O6) and a protonated N1-nitrogen, are also inactive in stimulating the editing reaction of ValRS (Table 3). However, previous studies showed that ValRS can deacylate mischarged Thr-tRNA^{Val}(G76) (posttransfer editing), although at a much slower rate than mischarged wild-type tRNA^{Val}(A76) (Tardif et al. 2001).

TABLE 3. Transfer RNA-dependent stimulation of editing by *E. coli* ValRS

Analog substitution	Rate of ATP hydrolysis (pmole/min) ^a	Relative rate of ATP hydrolysis	Steady-state level of misacylation with threonine (pmole/A ₂₆₀)
Adenosine	845	(1.0)	0
3-Deazaadenosine	823	0.97	6
7-Deazaadenosine	654	0.77	12
Purine Riboside	675	0.80	14
Inosine	17	0.02	153
2-Aminopurine	20	0.02	429
2,6-Diaminopurine	208	0.25	9
Xanthosine	10.2	0.01	34
Isoguanosine	169	0.20	381
7-Deazaguanosine	-48	-0.06	12
Uridine	-25	-0.03	1628
Cytidine	-88	-0.10	1312
Guanosine	-29	-0.03	36
No tRNA	-51	-0.06	—

^aNegative values are the result of small variations in the background rate of ATP hydrolysis.

An exocyclic amino group at position-2 of the 3'-terminal purine (guanine, 2-aminopurine, and 2,6-diaminopurine) reduces the ability of tRNA^{Val} to promote editing. *E. coli* tRNA^{Val} terminating in 3'-2-aminopurine riboside is inactive in stimulating ATP hydrolysis, and is stably misacylated with threonine to a level of more than 400 pmole/A₂₆₀ (Table 3). Introducing a second exocyclic amino group into the 3'-terminal purine of tRNA^{Val} by replacing the normal 3'-adenine (6-amino purine) with 3'-2,6-diaminopurine inhibits editing activity by 75% (Table 3); relatively little misacylation of this tRNA with threonine is observed (Table 3). Additional evidence of the inhibitory effect of a 2-NH₂ substituent on the 3'-terminal nucleotide is the observation that the editing activity of 3'-guanosine-substituted tRNA^{Val} is lower than that of the 3'-inosine-substituted tRNA (Table 3).

Experiments with *E. coli* tRNA^{Val} terminating with 3'-isoguanosine or 3'-xanthosine show that an O2 substituent inhibits the editing-stimulatory activity of the tRNA. Addition of a keto group at position-2 of the 3'-terminal adenine, yielding isoguanine, reduces editing activity of tRNA^{Val} by 80%, and consequently, 3'-isoguanosine-substituted tRNA^{Val} is misacylated with threonine to a level of almost 400 pmole/A₂₆₀ (Table 3). The tRNA^{Val} with a 3'-xanthosine substitution is less active than tRNA terminating in 3'-inosine (Table 3), presumably due to addition of the keto substituent at the 2-position.

Recognition of 3'-terminal hydroxyl groups by valyl-tRNA synthetase

To explore the role of 3'-terminal hydroxyl groups in aminoacylation and editing by ValRS, the 3'-adenosine of tRNA^{Val} was replaced with 2'-deoxyadenosine, 3'-deoxyadenosine, and 2', 3'-dideoxyadenosine (structures shown in Fig. 2). ValRS initially esterifies valine onto the 2'-hydroxyl group of the 3'-terminal ribose (Hecht and Chinault 1976). As expected, at high concentrations of ValRS (1–2 μM), 3'-deoxyadenosine-substituted tRNA^{Val} is charged with valine to the same level as wild-type tRNA^{Val} (Fig. 3A). At lower enzyme levels (1 nM), where the time course of valine acceptance can be more readily determined, substitution of the 3'-OH of tRNA^{Val} with a hydrogen significantly lowers aminoacylation activity. 3'-deoxyadenosine-substituted tRNA^{Val} is charged at a rate less than 20% that of wild-type tRNA^{Val} (Fig. 3B), suggesting that the 3'-hydroxyl group of the 3'-terminal nucleotide does contribute to tRNA recognition at the aminoacylation site of ValRS.

Derivatives of tRNA^{Val} with 3'-terminal 2'-deoxyA76, 3'-deoxyA76, and 2',3'-dideoxyA76 do not stimulate the tRNA-dependent editing reaction, as measured by ATP hydrolysis (Table 4), even at a

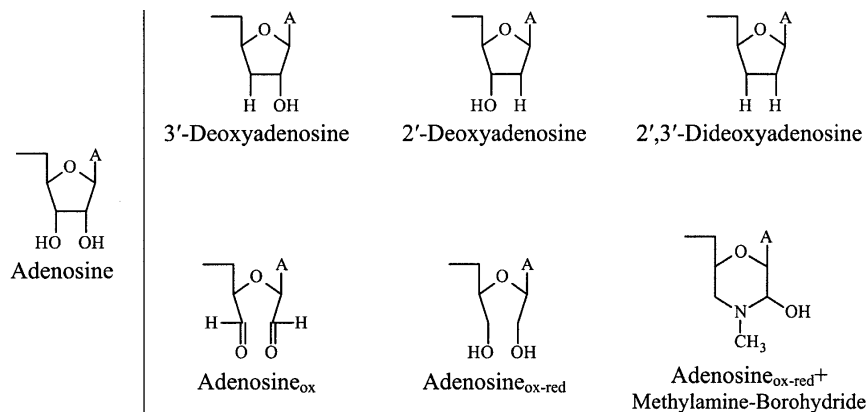


FIGURE 2. Chemical structures of the modified ribose derivatives substituted at the 3'-end of tRNA^{Val}.

high (5 μ M) tRNA concentrations (data not shown). As a result, 3'-deoxyA76 tRNA^{Val} is stably misacylated with threonine (Fig. 3A; Table 4). The maximum level of charging with threonine is, however, only about 25% that of valine acceptance (Fig. 3A; Table 4).

Although tRNA^{Val} terminating in 3'-deoxyA76 [tRNA^{Val} (3'dA76)] fails to stimulate editing (ATP hydrolysis) under our conditions, mischarged Thr-tRNA^{Val}(3'dA76) can be deacylated by ValRS (posttransfer editing; Fig. 3C). Absence of the terminal 3'-OH group, however, decreases the editing efficiency of the enzyme; the rate of deacylation of Thr-tRNA^{Val}(3'dA76) is much slower than that of misacylated wild-type Thr-tRNA^{Val}(3'rA76). Although we could not directly compare the deacylation rate of Thr-tRNA^{Val}(3'dA76) with that of Thr-tRNA^{Val}(3'rA76) because the latter is deacylated too rapidly by wild-type ValRS to be isolated, Nordin and Schimmel (2002), using an editing-deficient mutant of ValRS to prepare Thr-tRNA^{Val}(3'rA76), showed that it is deacylated 10 times more rapidly than Thr-tRNA^{Val}(3'dA76). The ability of ValRS to slowly hydrolyze Thr-tRNA^{Val}(3'dA76) explains the observed reduced levels of stable misacylation of this tRNA^{Val} variant with threonine (Fig. 3A; Table 4), because plateau charging levels represent a steady state resulting from competing aminoacylation and deacylation reactions.

Further evidence that absence of the 3'-OH inhibits recognition of aminoacyl-tRNA at the editing site of ValRS comes from examination of the deacylation of correctly charged valyl-tRNA^{Val}. Aminoacyl-tRNA synthetases are weak deacylases of their cognate aminoacyl-tRNAs (e.g., Schreier and Schimmel 1972; Igloi et al. 1977), and *E. coli* ValRS hydrolyzes correctly charged wild-type valyl-tRNA^{Val} (Fig. 3C). However, valyl-tRNA^{Val} lacking the 3'-OH [Val-tRNA^{Val}(3'dA76)] is not deacylated (Fig. 3C).

Our results indicating that the hydroxyl groups at the 3'-end of tRNA^{Val} play a significant role in the recognition of tRNA^{Val} at the aminoacylation and editing sites of ValRS and in minimizing ValRS-catalyzed misacylation of tRNA^{Val}

are corroborated by chemical modification of the 3'-terminal ribose of *E. coli* tRNA^{Val}. Periodate oxidation of the 3'-terminal ribose, to form the dialdehyde (see structures in Fig. 2) eliminates the valine-accepting activity (data not shown) and the editing-stimulatory activity of tRNA^{Val} (Table 4). Moreover, borohydride reduction of the oxidized tRNA^{Val} to yield primary hydroxyls at C2' and C3' of the 3'-terminal nucleotide (Fig. 2) does not restore the valine-accepting activity or the editing activity of the tRNA (Table 4). Tal et al. (1972) also observed that neither periodate-oxidized or oxidized-reduced tRNA^{Val} is able to accept valine, whereas several

other tRNAs recover accepting activity after the oxidized tRNA is reduced. Thus, whereas the integrity of the covalent bond between C2' and C3' of the 3'-terminal adenosine plays only a minor role in the aminoacylation of some tRNAs, it is clearly an important recognition element for the aminoacylation of *E. coli* tRNA^{Val} by ValRS. Finally, treatment of oxidized tRNA^{Val} with methylamine and borohydride chemically and structurally modifies the 3'-terminal ribose of tRNA^{Val} (Fig. 2), abolishing its aminoacylation and editing-stimulatory activity (Table 4).

DISCUSSION

The 3'-end of tRNA^{Val} plays an essential role in aminoacylation and editing by ValRS (see Introduction). It is correctly positioned at the synthetic and editing active sites of the enzyme by a variety of molecular interactions including hydrogen bonding between donor (d) and acceptor (a) groups on the terminal nucleotide and appropriately positioned amino acids, and by hydrophobic or stacking interactions with nearby protein side chains. Our previous results showed that replacing A76 of *E. coli* tRNA^{Val} with pyrimidines (U76 or C76) yields mutant tRNAs that can be actively aminoacylated (Liu and Horowitz 1994), but these tRNAs do not stimulate the tRNA-dependent editing reaction of ValRS (Table 3; Tardif et al. 2001). As a result, 3'-pyrimidine-substituted tRNA^{Val} is stably misacylated with threonine (Tamura et al. 1994; Liu et al. 1998) and several other noncognate amino acids (Tardif et al. 2001). Moreover, ValRS is unable to hydrolyze these misacylated tRNAs (posttransfer editing; Tardif et al. 2001). Conversely, although 3'-guanosine-substituted tRNA^{Val} [tRNA^{Val} (G76)] is aminoacylated only with difficulty (Liu and Horowitz 1994; see Table 1), misacylated tRNA^{Val}(G76) is hydrolyzed by ValRS (Tardif et al. 2001). Evidently, only 3'-terminal purines are recognized at the editing site, whereas the aminoacylation site recognizes pyrimidines as well as adenine. Proteins frequently recognize adenine

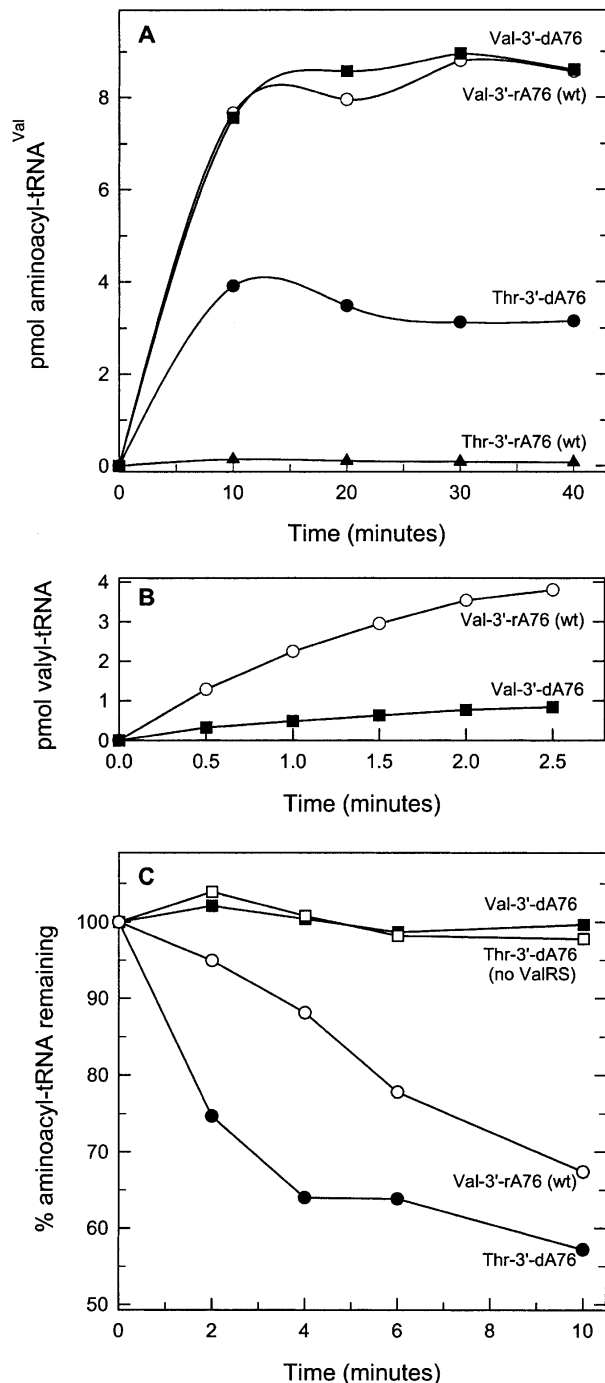


FIGURE 3. (A) Aminoacylation and misacylation of wild-type and 3'-deoxyribose-substituted tRNA^{Val}: wild-type valyl-3'-rA76 (○), valyl-3'-dA76 (■), threonyl-3'-dA76 (●), threonyl-3'-rA76 (▲). ValRS concentration is 1.5 μM. (B) Time course of aminoacylation of wild-type and 3'-deoxyribose-substituted tRNA^{Val} with valine: wild-type 3'-rA76 (○), 3'-dA76 (■). ValRS concentration is 1 nM. (C) Posttransfer editing by valyl-tRNA synthetase: wild-type valyl-3'-rA76 (○), valyl-3'-dA76 (■), threonyl-3'-dA76 (●), No ValRS (with threonyl-3'-dA76) (□). ValRS and aminoacyl-tRNA concentrations were 0.3 μM and 2 μM, respectively.

through H-bond interactions between amino acid side chains and the proton acceptor (a) N1 and the proton donor (d) N6 sites (Fig. 4). A similar \bullet •d pattern of hydrogen bond donor and acceptor groups is present in cytidine and uridine, but not in guanosine (Fig. 4). This may explain why cytidine and uridine, but not guanosine, can functionally substitute for A76 of tRNA^{Val} in the aminoacylation reaction of ValRS, and suggests that the exocyclic amino group (N6) and/or the ring N1 of 3'-terminal adenosine are specifically recognized at the synthetic (aminoacylation) active site.

Such hydrogen bonding has been observed at the editing active site of LeuRS (like ValRS a class Ia aminoacyl-tRNA synthetase), where the N1 and N6 positions of a nonhydrolyzable sulfamoyl analog of norvalyl-adenylate (pretransfer editing complex) and of an analog of the 3'-terminal adenosine of norvalyl-tRNA (posttransfer editing complex) are both H-bonded to the α -NH and α -CO groups, respectively, of Leu³²⁹, and the adenine ring stacks on a conserved hydrophobic amino acid side chain (Ile³³⁷; Lincecum et al. 2003). The recently solved crystal structure of *Thermus thermophilus* ValRS, complexed with tRNA^{Val} (with its 3'-end at the editing site) and a Val-AMP analog (Fukai et al. 2000), shows that the N1 atom and 6-NH₂ group of A76 form H-bonds with the α -NH and α -CO groups, respectively, of Glu²⁶¹. The purine ring of A76 is sandwiched between the side chains of Phe²⁶⁴ and Leu²⁶⁹ by van der Waals contacts. Such an extensive network of H-bonds and hydrophobic interactions emphasizes the importance of a purine, particularly adenosine, at the 3'-end of tRNA and would explain why substitution of A76 has a major perturbing effect on the editing reaction.

Although these results suggest that specific recognition of the exocyclic amino group (N6) of the 3'-terminal adenosine is important for function, our base substitution experiments demonstrate that the N6 substituent is not a positive determinant for tRNA recognition at the synthetic (aminoacylation) site of *E. coli* ValRS; the same is true for the N3 and N7 ring nitrogens of A76. Variants of tRNA^{Val} terminating in 3'-purine riboside, which lacks the exocyclic N6 amino group, or in 3'-terminal 3-deazaadenosine or 7-deazaadenosine, which have, respectively, their N3 and N7 ring nitrogens replaced by carbons, retain almost full aminoacylation activity (Table 1).

Whereas the exocyclic amino group of adenosine is not important for recognition of the 3'-end of tRNA^{Val} at the synthetic active site of ValRS, an O6 substituent interferes with aminoacylation. *E. coli* tRNA^{Val} terminating in 3'-guanosine, 3'-inosine, or 3'-xanthosine is a poor substrate for aminoacylation by ValRS (Table 1). Either the 6-keto group acts as an antideterminant or, more likely because ValRS does not recognize substituents at the 6-position, changing the protonation state of N1 from an H-bond acceptor to a hydrogen bond donor (Fig. 4) inhibits function. The requirement for an unprotonated N1 may be a major positive

TABLE 4. Stimulation of editing by *E. coli* tRNA^{Val} with modified 3'-terminal hydroxyl groups

3'-Modified tRNA ^{Val}	Rate of ATP hydrolysis (pmole/min) ^a	Relative rate of ATP hydrolysis	Steady-state level of misacylation with threonine (pmole/A ₂₆₀)
Ribose derivative			
Adenosine	745	(1.0)	10
3'-Deoxyadenosine	-14	-0.02	408
2'-Deoxyadenosine	1.8	0	151
2',3'-Dideoxyadenosine	48	0.06	163
tRNA ^{Val} (-A)	-33	-0.04	ND ^b
No tRNA	-0.07	0	—
Chemically modified ribose			
Adenosine _{ox}	-8.0	-0.03	ND
Adenosine _{ox-red}	-1.3	-0.004	ND
Adenosine _{ox-red} + methylamine-borohydride	-9.6	-0.03	ND
No tRNA	-3.7	-0.01	ND

^aNegative values are the result of small variations in the background rate of ATP hydrolysis.

^bND, not determined.

determinant for recognition of the 3'-terminal nucleotide of tRNA^{Val}.

Substituents at the 2-position are antideterminants for recognition of the 3'-terminal purine of tRNA^{Val}, largely due to a decrease in k_{cat} (Table 2). The aminoacylation activity of 2-aminopurine-substituted tRNA^{Val} is lower than that of the purine riboside-substituted tRNA, as is that of the guanosine-substituted tRNA compared to 3'-inosine-

terminating in 3'-inosine is higher than that of the tRNA terminating in 3'-xanthosine (Table 1), again the result of the O2 substituent present in the latter.

The 3'-terminal hydroxyl groups of tRNA^{Val} also contribute to tRNA recognition by the synthetic site of ValRS. *E. coli* tRNA^{Val} terminating with 3'-deoxyadenosine can be fully aminoacylated, as expected, because ValRS is known to

esterify the 2'-hydroxyl group (Hecht and Chinault 1976). This tRNA^{Val} variant, however, is charged at only 17% the rate of wild-type tRNA^{Val} (Fig. 3B), demonstrating that the 3'-hydroxyl group improves the aminoacylation efficiency of the enzyme. Furthermore, an intact covalent bond between C2' and C3' of the 3'-terminal adenosine is important for aminoacylation of tRNA^{Val} by ValRS, because *E. coli* tRNA^{Val} terminating in adenosine_{ox-red} is not aminoacylated by the enzyme (Table 4).

Because our results indicate that transient misacylation of tRNA is a necessary prelude to editing (Tardif and Horowitz 2002; also see later discussion), it is difficult to identify functional groups specifically recognized at the editing site. However, because replacing A76 with purine riboside, 7-deazaA, and 3-deazaA has little or no effect on aminoacylation or total editing (Tables 1, 3), we can conclude that the exocyclic 6-amino group and the N7 and N3 ring

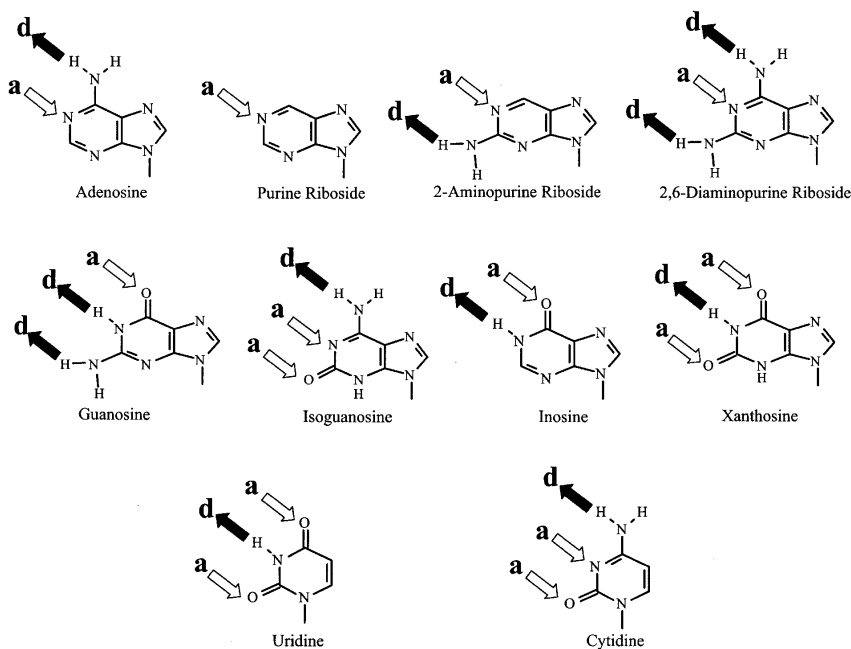


FIGURE 4. Hydrogen bond acceptor (a) and donor (d) sites of purine and pyrimidine bases. Filled arrows, donor groups; open arrows, acceptor sites.

nitrogens of A76 are not essential for triggering the editing reaction of ValRS. Furthermore, we have shown (Tardif et al. 2001) that ValRS deacylates mischarged Thr-tRNA^{Val} (G76) (posttransfer editing) at a much slower rate than mischarged wild-type (A76) tRNA^{Val}. Either the editing site preferentially recognizes an unprotonated N1, or the NH₂ substituent at position-2 acts as a negative determinant, or both.

In addition to enhancing tRNA recognition at the aminoacylation site of ValRS, the 3'-hydroxyl group of A76 is a recognition element for translational editing. 3'-deoxy-adenosine-substituted tRNA^{Val}, misacylated with threonine on the 2'-OH, is deacylated by ValRS (posttranslational editing; Fig. 3B; Nordin and Schimmel 2002) but at a rate 10 times slower than that of misacylated wild-type Thr-tRNA^{Val}(3'rA76) (Nordin and Schimmel 2002). Analysis of the cocrystal structure of *T. thermophilus* ValRS with its cognate tRNA (Fukai et al. 2000) and that of the closely related *T. thermophilus* LeuRS/tRNA^{Leu} complex (Lincecum et al. 2003) provides insight into the structural role of the 3'-OH in promoting editing (the structure of the *E. coli* ValRS/tRNA^{Val} complex has not been solved, and the 3'-end of the tRNA is not resolved in the structure of the *T. thermophilus* IleRS/tRNA complex). In both structures, a conserved threonine-rich sequence in the editing (CP1) domain, including Thr²¹⁴ and Val²¹⁵ in *T. thermophilus* ValRS (corresponding to Thr²²¹ and Thr²²² in *E. coli* ValRS) and Thr²⁴⁷ and Thr²⁴⁸ in *T. thermophilus* LeuRS (Lincecum et al. 2003), interacts with the hydroxyl groups of A76. The *T. thermophilus* ValRS cocrystal structure shows hydrogen bonds between the side chain OH of Thr²¹⁴ and the 2'-OH of A76 and between the 3'-OH of A76 and the peptide NH of Val²¹⁵ (Fukai et al. 2003). Molecular modeling studies, using Swiss-PdbViewer (Guex and Peitsch 1997) to convert Val²¹⁵ of *T. thermophilus* ValRS to a Thr residue so that the sequence corresponds to that in *E. coli* ValRS, demonstrate that Thr²¹⁵ can make two H-bonds to the 3'-OH of the ribose of A76 (data not shown). This is similar to the structure of the *T. thermophilus* LeuRS complex with a posttransfer editing analog, where the 3'-OH of the A makes three H-bonds with Thr²⁴⁷ and Thr²⁴⁸ (corresponding to Thr²¹² and Thr²²² in *E. coli* ValRS). Such hydrogen bond interactions with the 3'-OH may serve to properly orient the mischarged tRNA in the editing site and thus explain the contribution of the 3'-OH to the efficiency of editing.

Our results regarding the effects of replacing the universally conserved 3'-adenosine of *E. coli* tRNA^{Val} with nucleoside analogs that have a variety of substituents on the purine and ribose rings support and extend previous findings (Horowitz et al. 1999; Tardif and Horowitz 2002) that there is a close correlation between the aminoacylation efficiency of a tRNA and its effectiveness in stimulating the editing reaction of ValRS (Tables 1, 3). These results, together with our earlier findings (Tardif et al. 2001), and those of Nordin

and Schimmel (2003), showing that misacylated tRNA^{Val} variants that cannot be deacylated fail to promote overall editing by ValRS, strongly suggest that misacylated tRNA^{Val} is a transient intermediate in the editing reaction, that is, that editing by ValRS requires a posttransfer step.

The question of which editing pathway, pre- or post-transfer, predominates has been extensively investigated for IleRS and ValRS. Based on comparison of the cocrystal structures of the homologous IleRS and ValRS complexed with a variety of substrates, and on modeling predictions, distinct but proximal subsites for binding the amino acids of pretransfer and posttransfer editing substrates were proposed, each involving a separate set of main chain amino acids (Nureki et al. 1998; Fukai et al. 2000). Separate sites would explain the ability of the tRNA synthetases to hydrolyze two very different substrates, a mixed anhydride (pre-transfer editing) and an aminoacyl ester (posttransfer editing).

A model for editing by IleRS was recently proposed (Bishop et al. 2002) that explains many previous observations, including the finding that mutation of D342 in *E. coli* IleRS greatly diminishes total editing (pre- plus posttransfer) even though the Fukai two-subsite editing model indicates that this amino acid is part of the posttransfer editing site of IleRS but not of the pretransfer editing site. Bishop et al. (2002) suggested that the IleRS/tRNA^{Ile} complex catalyzes one round of posttransfer editing to prime the enzyme for subsequent rounds of pretransfer editing. The model implies that the 3'-end of tRNA and the adenylate moiety of the aminoacyl-adenylate can bind simultaneously to the editing site. One difficulty with this model, as Bishop et al. point out, is that pretransfer editing requires that IleRS successively misactivate two or more molecules of the non-cognate amino acid, valine. This seems unlikely in vivo, because the enzyme binds the cognate isoleucine ~100 times more tightly than valine.

Recent results with LeuRS (Lincecum et al. 2003) shed new light on the editing mechanism. ValRS, IleRS, and LeuRS represent a closely related subfamily of class Ia aminoacyl-tRNA synthetases. All are large (100-kD) monomers and contain a sizable CP1 insert responsible for the editing reaction. Although there are some structural differences among the three enzymes (Cusack et al. 2000), their overall structures are quite similar (Nureki et al. 1998; Cusack et al. 2000; Fukai et al. 2000; Lincecum et al. 2003). The high structural and sequence homologies among the editing sites of these enzymes suggest that they may share a similar editing mechanism (Lincecum et al. 2003).

High-resolution crystallographic structures of LeuRS bound to pre- and posttransfer editing substrate analogs indicate that both bind to the same or largely overlapping sites in the editing domain (Lincecum et al. 2003). Analysis of these structures shows that the conformation of the ribose moiety of the pretransfer substrate differs from that of the posttransfer substrate, allowing the adenine ring and the

amino acid of each to be recognized by a single editing site (Lincecum et al. 2003), which is able to hydrolyze both the pretransfer substrate (an aminoacyl-adenylate) and the posttransfer substrate (an aminoacyl-ester). This result is inconsistent with the two-subsite editing model for IleRS and ValRS proposed by Fukai et al. (2000), in which the amino acid of the pre- and posttransfer substrates is bound at different subsites of the editing domain. A possible reason for the difference is that in modeling threonyl-adenylate (pretransfer substrate) at the observed location of the 3'-terminal adenosine of tRNA^{Val} (posttransfer substrate) at the editing site of ValRS, Fukai et al. assumed that the conformation of the ribose in the pre- and posttransfer substrates is identical, which is not the case for LeuRS. A consequence of the results with LeuRS, showing that the enzyme has a single editing pocket, is that the 3'-end of tRNA and the adenylate portion of the aminoacyl adenylate cannot simultaneously bind to the editing site as implied in the model for editing proposed by Bishop et al. (2002). Further studies will be required to determine whether the same or different editing mechanisms are operative in all three synthetases.

MATERIALS AND METHODS

Materials

Restriction endonucleases were purchased from either New England Biolabs or Promega. T4 RNA ligase and calf intestinal phosphatase were from New England Biolabs; inorganic pyrophosphatase was a product of Boehringer Mannheim Biochemicals. Homogeneous valyl-tRNA synthetase was prepared from *E. coli* GRB238/pHOV1 by the procedure of Chu and Horowitz (1991). T7 RNA polymerase was isolated from *E. coli* BL21/pAR1219 as reported by Zawadzki and Gross (1991). ATP(CTP):tRNA nucleotidyltransferase was purified from *E. coli* BL21(DE3) (kindly provided by Drs. N. Maizels and A.M. Weiner, University of Washington, Seattle) according to Shi et al. (1998).

Nucleotide triphosphates and guanosine 5'-monophosphate were the products of Sigma, Amersham Life Sciences, or United States Biochemical. Modified nucleosides: isoguanosine, 7-deazaadenosine, 7-deazaguanosine, purine riboside, inosine, 2,6-diaminopurine, and 2-aminopurine, were obtained from ChemGenes; 3-deazaadenosine was purchased from Southern Research Institute and xanthosine from Sigma. [³H]valine (23–32 Ci/mmol), [³H]threonine (15.8 Ci/mmol), and [γ -³²P]adenosine-5'-triphosphate (3 Ci/mmol) were from Amersham Life Sciences.

Preparation and characterization of nucleoside bisphosphates

Nucleoside bisphosphates were synthesized by the method of Barrio et al. (1978) and chromatographically purified on a DEAE Sephacel column (30 × 1.5 cm) equilibrated with 50 mM TEAB (pH 8.0). The column was developed with a linear gradient from 50 mM to 400 mM TEAB (pH 8.0). This method yields mixtures of the 2', 5'- and 3', 5'-bisphosphates which were not separated

because the 2', 5'-bisphosphates do not interfere with ligation of the 3', 5'-bisphosphates with tRNA (Barrio et al. 1978). Purified nucleoside bisphosphates (50 A₂₆₀) were characterized by ³¹P NMR (Table 5) in a solution containing 0.84 M tetramethylammonium hydroxide (pH 10), 2 mM EDTA, 20% D₂O. ³¹P NMR spectra were collected on a Bruker AC-200 spectrometer.

Preparation of tRNA

Wild-type tRNA^{Val} was transcribed in vitro by T7 RNA polymerase as reported previously (Chu and Horowitz 1989) from a DNA template derived from the recombinant phagemid pFVAL119, which contains a T7 promoter directly upstream from a tRNA^{Val} gene and a *FokI* restriction site directly downstream of the tRNA^{Val} gene (Liu and Horowitz 1993). Transcripts were purified by HPLC (Liu and Horowitz 1993). Valine tRNA lacking the 3'-terminal adenosine [tRNA^{Val}(-A)] was transcribed from a deletion mutant of pFVAL119 prepared by PCR site-directed mutagenesis using the QuikChange kit (Stratagene) as described by the manufacturer. The mutagenic oligonucleotides 5'-CATCACCCACCGGATC CAGTCATC-3' and its complement were synthesized by the Nucleic Acid Facility at Iowa State University. Mutants were selected by restriction endonuclease digestion patterns, and the sequence was confirmed by automated dideoxy sequence analysis performed by the Nucleic Acid Facility.

Preparation of 3'-end-substituted tRNA^{Val}

Valine tRNAs with modified bases at the 3'-end were prepared by ligating nucleoside bisphosphates to the 3'-terminus of tRNA^{Val} lacking the 3'-terminal adenosine [tRNA^{Val} (-A)] with T4 RNA ligase, as described by Paulsen and Wintermeyer (1984). 3'- (and 5')-terminal phosphates were removed with calf intestinal phosphatase in a reaction mixture containing 50 mM Tris-HCl (pH 8.0), 8 mM MgCl₂, 2 u/μL calf intestinal phosphatase, and 0.8 μg/μL tRNA. Modified RNAs were separated from unligated start-

TABLE 5. ³¹P chemical shifts of nucleoside 3'(2'), 5'-bisphosphates

Nucleoside bisphosphate	Chemical shift ^a		
	3'-P	2'-P	5'-P
pAdenosine3'p	4.883	—	4.589
pAdenosine3'(2')p	4.885	4.302	4.590
pGuanosine3'(2')p	4.922	4.134	4.577
p2,6-Diaminopurine3'(2')p	4.995	4.284	4.643
p2-Aminopurine3'(2')p	4.961	4.218	4.661
pIsoguanosine3'(2')p	4.943	4.016	4.644
pPurine riboside3'(2')p	5.127	4.331	4.637
pInosine3'(2')p	4.971	4.205	4.612
p7-Deazaadenosine3'(2')p	5.016	4.022	4.607
p7-Deazaguanosine3'(2')p	4.973	3.997	4.561
p3-Deazaadenosine3'(2')p	5.039	4.278	4.669
pXanthosine3'(2')p	4.893	3.930	4.457
pCytidine3'(2')p	4.806	4.312	4.556

^aSpectra were recorded in 0.84 M tetramethylammonium hydroxide (pH 10), 2 mM EDTA, 20% D₂O.

ing RNA by electrophoresis on 8% denaturing polyacrylamide gels (20 × 45 × 0.2 cm) for 18 h at a constant current of 15 mA. RNA was extracted from the gel with 0.5 M NH₄(OAc), 10 mM Mg(OAc)₂, 1 mM EDTA (pH 8.0), and 0.1% SDS, using the crush and soak method (Maniatis et al. 1989). Gel-purified RNAs were ethanol-precipitated and dissolved in 10 mM Tris-HCl (pH 7.4), 10 mM MgCl₂.

Because of difficulties synthesizing nucleoside bisphosphates of adenine nucleosides having sugar modifications, valine tRNAs with modified sugars were prepared by ligating the nucleoside triphosphates ATP, 2'-dATP, 3'-dATP, and 2',3'-ddATP to tRNA^{Val} (-A) with *E. coli* nucleotidyltransferase, as described by Francis et al. (1983). The resulting modified tRNAs were purified by chromatography on a Toyopearl DEAE-650S HPLC column (Liu and Horowitz 1993), followed by electrophoretic purification as described previously.

The 3'-terminal ribose of tRNA^{Val} was chemically modified by periodate oxidation, reduction with sodium borohydride, and reaction with methylamine, as described by Fahnestock and Nomura (1972).

Aminoacylation assays

Maximum (plateau) aminoacylation levels of tRNA transcripts were determined at 37°C as described (Kintanar et al. 1994) in a 60-μL reaction mix containing 100 mM HEPES-KOH (pH 7.5), 10 mM KCl, 15 mM MgCl₂, 7 mM ATP, 1 mM DTT, 100 μM L-[³H]valine (23–32 Ci/mmol), 1–4 μg of tRNA, and 2–4 μg of ValRS (an excessive amount). Levels of misacylation with threonine were assayed as described for valine, with a ValRS concentration of 1.5 μM and 83 μM L-[³H]threonine (15.8 Ci/mmol). The time course of aminoacylation, under k_{cat}/K_m conditions, was followed in reactions using 1 nM ValRS and a low (1 μM) tRNA concentration. Steady-state aminoacylation kinetics, to determine individual k_{cat} and K_m values (Table 2), were measured under similar conditions with transfer RNA concentrations ranging from 0.5 to 6.0 μM. Reactions were initiated by addition of 1 nM of purified ValRS. K_m and k_{cat} values were calculated from a least-squares fit of the double reciprocal plot of the data using the Enzfitter computer program (Elsevier-Biosoft). Values reported are the average of two or more experiments.

Editing assay

Total editing activity (pre- plus posttransfer) was determined by an ATP hydrolysis assay essentially as described by Schmidt and Schimmel (1994). Reaction mixtures of 60 μL containing 150 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 37 mM threonine, 3 mM [γ -³²P]ATP (20 to 30 cpm/pmol), 1 μM tRNA, and 2 μM ValRS were incubated at 25°C. At intervals over 30 min, 10 μL of the reaction was quenched with 25 volumes of 7% HClO₄, 10 mM sodium pyrophosphate, and 3% activated charcoal (Sigma). [³²P]pyrophosphate released as a result of ATP hydrolysis was separated from charcoal-bound ATP/AMP by centrifugation. Radioactivity in a 50-μL sample of the supernatant was determined by liquid scintillation counting. Results reported are the average of two or more determinations. Under conditions of the experiment, the rate of ATP hydrolysis is proportional to the concentration of tRNA (Tardif and Horowitz 2002).

Deacylation of aminoacyl-tRNA (posttransfer editing)

To measure the rate of deacylation of aminoacylated tRNA (post-transfer editing), the RNA was aminoacylated, as described above, with a [³H]-labeled amino acid and purified by chromatography on Sephadex G-25 (coarse grade, Pharmacia) at pH 5.0 (5 mM potassium acetate) to remove residual free radioactive amino acid. Purified aminoacyl-tRNAs were stored in 5 mM potassium acetate (pH 5.0).

Deacylation of aminoacyl-tRNA was followed as described by Lin and Schimmel (1996) at 37°C in a 60-μL reaction mixture containing 150 mM Tris-HCl (pH 7.5), 150 mM KCl, 10 mM MgCl₂, and 2 μM [³H]-labeled aminoacyl-tRNA. The reaction was started by addition of 0.3 μM ValRS. Samples of 10 μL were removed at the indicated times, and remaining labeled aminoacylated tRNA was determined (Liu et al. 1998). Spontaneous hydrolysis of the aminoacyl bond was followed under the same conditions in the absence of ValRS.

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