Misacylation and Editing by *Escherichia coli* Valyl-tRNA Synthetase: Evidence for Two tRNA Binding Sites†

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ABSTRACT: Valyl-tRNA synthetase (ValRS) has difficulty discriminating between its cognate amino acid, valine, and structurally similar amino acids. To minimize translational errors, the enzyme catalyzes a tRNA-dependent editing reaction that prevents accumulation of misacylated $tRNA^{Val}$. Editing occurs with threonine, alanine, serine, and cysteine, as well as with several nonprotein amino acids. The 3′-end of tRNA plays a vital role in promoting the tRNA-dependent editing reaction. Valine tRNA having the universally conserved 3′-terminal adenosine replaced by any other nucleoside does not stimulate the editing activity of ValRS. As a result 3′-end $tRNA^{Val}$ mutants, particularly those with 3′-terminal pyrimidines, are stably misacylated with threonine, alanine, serine, and cysteine. Valyl-tRNA synthetase is unable to hydrolytically deacylate misacylated $tRNA^{Val}$ terminating in 3′-pyrimidines but does deacylate mischarged $tRNA^{Val}$ terminating in adenosine or guanosine. Evidently, a purine at position 76 of $tRNA^{Val}$ is essential for translational editing by ValRS. We also observe misacylation of wild-type and 3′-end mutants of $tRNA^{Val}$ with isoleucine. Valyl-tRNA synthetase does not edit wild-type $tRNA^{Val}$(A76) mischarged with isoleucine, presumably because isoleucine is only poorly accommodated at the editing site of the enzyme. Misacylated mutant tRNAs as well as 3′-end-truncated $tRNA^{Val}$ are mixed noncompetitive inhibitors of the aminoacylation reaction, suggesting that ValRS, a monomeric enzyme, may bind more than one $tRNA^{Val}$ molecule. Gel-mobility-shift experiments to characterize the interaction of $tRNA^{Val}$ with the enzyme provide evidence for two tRNA binding sites on ValRS.

The accuracy of protein synthesis depends on the aminoacylation of transfer RNA with its correct amino acid. Some aminoacyl-tRNA synthetases, however, have difficulty in discriminating their cognate amino acid from structurally similar ones and misactivate noncognate amino acids. To minimize errors and maintain translational fidelity, these enzymes catalyze proofreading (editing) reactions at either of two steps in the aminoacylation process (reviewed in ref *1*): by hydrolyzing the enzyme-bound misactivated aminoacyl adenylate (pretransfer editing) or by deacylating enzyme-bound misacylated tRNA (posttransfer editing). Both reactions are stimulated by the presence of cognate tRNA (*2*, *3*).

A number of class I aminoacyl-tRNA synthetases catalyze editing reactions, including IleRS (*4*, *5*), ValRS[1] (*2*, *3*), MetRS (*3*), and LeuRS (*6*), all members of the same subgroup of class I aminoacyl-tRNA synthetases. Although the editing reactions of class II synthetases have been less intensively studied, PheRS (*7*, *8*), AlaRS (*9*), LysRS (*10*, *11*), ThrRS (*12*), and ProRS (*13*) are thus far known to catalyze editing. Mutational analysis of IleRS (*14*, *15*) and ValRS (*15*, *16*) has shown that the aminoacylation and editing reactions occur at functionally independent sites; a large insert in the active site (connective polypeptide 1 or CP1) is responsible for the editing activity (*15*, *17*−*20*). Cognate tRNAs specifically trigger transfer of the misactivated amino acid from the activation site to the editing site, accounting for the tRNA dependence of the editing reaction (*19*, *20*).

Several previous studies have examined the role of the universally conserved 3′-CCA end of tRNA in aminoacylation and translational editing. Chemical and enzymatic modifications of the 3′-terminal adenosine (*21*−*24*) or its ribose moiety (*25*) result in total loss or dramatic decreases in the ability of the tRNA to accept amino acids, due largely to a decrease in the k_{cat} of the reaction. Some of these modified tRNAs can be misacylated, suggesting that the 3′-terminal nucleotide is specifically recognized at the editing site of the synthetase. The contribution of the 3′-CCA

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[1] Abbreviations: ValRS, valyl-tRNA synthetase; $tRNA^{Val}$(−CCA), $tRNA^{Val}$ lacking the 3′-CCA sequence; $tRNA^{Val}$(−ACCA), $tRNA^{Val}$ lacking the 3′-ACCA sequence; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography.

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sequence of *Escherichia coli* $tRNA^{Val}$ to aminoacylation and editing by ValRS was studied by systematically mutating the 3′-terminal sequence (*26*, *27*). Most of these variants of in vitro transcribed $tRNA^{Val}$ are readily aminoacylated; only the tRNAs terminating in 3′-CCG and 3′-CGA are poor substrates for aminoacylation by ValRS (*26*). Tamura et al. (*27*) carried out similar studies with $tRNA^{Val}$ and found comparable results. These authors also observed that replacing the 3′-terminal adenine of $tRNA^{Val}$ with cytosine or uracil results in appreciable levels of misacylation with threonine.

This paper extends studies of misacylation and editing by *E. coli* ValRS. We describe the range of amino acid substrates the enzyme is able to misactivate and charge to $tRNA^{Val}$ and the role of the 3′-terminal base in the proofreading reaction catalyzed by ValRS. Inhibition studies show that misacylated and 3′-end-truncated valine tRNAs are mixed noncompetitive inhibitors of aminoacylation, and gel-mobility-shift experiments provide evidence for two tRNA binding sites on the monomeric enzyme.

MATERIALS AND METHODS

Materials. Restriction endonucleases were purchased from either New England Biolabs or Promega. Inorganic pyrophosphatase was from Boehringer Mannheim Biochemicals. T7 RNA polymerase was isolated from *E. coli* BL21/pAR1219 as reported by Zawadzki and Gross (*28*).

Homogeneous ValRS was prepared from *E. coli* GRB238/pHOV1 by the procedure of Chu and Horowitz (*29*). Mobility-shift assays were performed with N-terminal His-tagged ValRS prepared from *E. coli* BL21(DE3) transformed with pValRS(His), which was prepared by inserting the ValRS gene into pET-16b (Novagen) (D. Spielbauer and J. Horowitz, unpublished). His-tagged ValRS was purified by chromatography on a cobalt immobilized metal affinity TALON resin (ClonTech).

Nucleoside triphosphates, guanosine 5′-monophosphate, [γ-^{32}P]adenosine 5′-triphosphate (3Ci/mmol), and [α-^{32}P]-adenosine 5′-triphosphate (3000 Ci/mmol) were the products of Sigma, Amersham Life Sciences, NEN, or U.S. Biochemical Co. [^{14}C]Valine (158 Ci/mol) and [^{14}C]isoleucine (342 Ci/mol) were purchased from ICN Biomedicals. [^{14}C]-Glutamic acid (53 mCi/mmol), [^{14}C]methionine (41 mCi/mol), [^{3}H]valine (23−32 Ci/mmol), [^{3}H]threonine (15.2 Ci/mmol), [^{3}H]alanine (50 Ci/mmol), [^{3}H]leucine (46 Ci/mmol), [^{3}H]lysine (40 Ci/mmol), [^{3}H]isoleucine (113 Ci/mmol), [^{3}H]-phenylalanine (54 Ci/mmol), [^{3}H]glycine (19 Ci/mmol), [^{3}H]-serine (28 Ci/mmol), [^{3}H]tyrosine (58 Ci/mmol), and [^{35}S]-cysteine (1200 Ci/mmol) were from Amersham Life Sciences.

Preparation of tRNA. Wild-type $tRNA^{Val}$ was transcribed in vitro by T7 RNA polymerase as reported previously (*30*) from a DNA template derived from the recombinant phagemid pFVAL119, which contains a T7 promoter directly upstream from a $tRNA^{Val}$ gene and a *Fok*I restriction site directly downstream of the $tRNA^{Val}$ gene (*31*). Transcripts were purified by HPLC (*32*). Mutations and deletions were introduced into the cloned tRNA genes by site-directed mutagenesis (*30*), using mutagenic oligonucleotides synthesized by the Nucleic Acid Facility at Iowa State University. The sequence of all mutants was confirmed by automated dideoxy sequence analysis performed by the Nucleic Acid Facility.

ATP Hydrolysis Assay. ATP hydrolysis assays were performed essentially as described by Schmidt and Schimmel (*14*). Reaction mixtures of 60 μL containing 150 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 37 mM amino acid, 3 mM [γ-^{32}P]ATP (20−30 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% $HClO_4$, 10 mM sodium pyrophosphate, and 3% activated charcoal (Sigma). [^{32}P]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. The rate of ATP hydrolysis reported is the average of two or more determinations.

Aminoacylation Assays. Maximum (plateau) aminoacylation levels of tRNA transcripts were determined at 37 °C as previously described (*32*) in a 60 μL reaction mix containing 100 mM HEPES−KOH (pH 7.5), 10 mM KCl, 15 mM $MgCl_2$, 7 mM ATP, 1 mM DTT, 100 μM radiolabeled amino acid, 1−4 μg of tRNA, and 2−4 μg of ValRS (an excessive amount). The time course of aminoacylation was followed in reactions using 1 nM ValRS and 2 μM tRNA. Aminoacylation kinetics 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 purified ValRS. K_m and V_{max} 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.

Studies of the inhibition of aminoacylation by aminoacyl-tRNAs were carried out at pH 6.5 to minimize spontaneous hydrolysis of the aminoacyl bond. Inhibitor concentrations in these experiments ranged from 0 to 20 μM. A statistical analysis MINITAB program (from Dr. Herbert Fromm, Iowa State University) was used to analyze the initial rate data. The program determines whether the results best fit a competitive or noncompetitive inhibition model based on which gives the smallest differences in the residuals.

Deacylation of Aminoacyl-tRNA. To measure the rate of deacylation of aminoacylated tRNA, the RNA was aminoacylated, as described above, with a ^{3}H-labeled amino acid. Although it is difficult to prepare threonyl-$tRNA^{Val}$ terminating in a 3′ G, sufficient amounts for the deacylation experiments can be obtained by carrying out the aminoacylation reaction at low ValRS concentrations (0.046 μM; see Table 3). Aminoacylated tRNAs were purified by chromatography on Sephadex G-25 (coarse grade, Pharmacia, Inc.) 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 (*16*) at 37 °C in a 60 μL reaction mixture containing 150 mM Tris-HCl (pH 7.5), 150 mM KCl, 10 mM $MgCl_2$, and 2 μM ^{3}H-labeled aminoacyl-tRNA. The reaction was started by addition of 30−1800 nM ValRS. Samples of 10 μL were removed at the indicated times, and the remaining labeled aminoacylated tRNA was determined (*33*). Spontaneous hydrolysis of the aminoacyl bond was followed under the same conditions in the absence of ValRS.

Identification of Amino Acid Misacylated on $tRNA^{Val}$. [^{14}C]-Isoleucyl-$tRNA^{Val}$ and [^{14}C]valyl-$tRNA^{Val}$ were prepared as described previously. These ^{14}C-labeled tRNAs, 200 pmol,

were incubated separately at 42 °C for 2 h in a 6 μL reaction mix containing 23 mM NaOH (pH 13), to strip the amino acid from the tRNA. Each reaction mix was then spotted at the origin of a cellulose TLC plate (EM Science), and the plates were developed a distance of 6.5 cm with a butanol−acetone−triethylamine−water (10:10:2:5 v/v/v/v) mixture. The location of amino acids was determined by autoradiography using Kodak X-OMAT AR film and the amino acids identified by comparison to the mobilities of authentic [^{14}C]-isoleucine and [^{14}C]valine (Amersham) chromatographed on the same TLC plate.

Gel-Retardation Experiments. Protein−tRNA interactions were examined by a gel-shift assay. T7 RNA polymerase transcripts of wild-type *E. coli* tRNA^{Val}, internally labeled with [α-^{32}P]AMP by transcription in the presence of [α-^{32}P]-ATP, were purified from 12% polyacrylamide−7 M urea gels. A small, constant amount of ^{32}P-labeled tRNA^{Val} and varying amounts of unlabeled tRNA^{Val} were combined with His-tagged *E. coli* ValRS in 10 μL of 5 mM DTT, 25 mM sodium acetate, pH 5.8, 10 mM KCl, and 10 mM $MgCl_2$ (*34*). The total concentration of tRNA^{Val} varied from 0 to 40 μM as did the concentration of ValRS. The total tRNA plus ValRS concentration was maintained constant at 40 μM. After incubation at room temperature for 15 min, the reactions were stopped by addition of 0.2% glutaraldehyde, which inactivates the enzyme, and 40% glycerol and electrophoresed on an 8% native polyacrylamide gel in the reaction buffer for 3 h at 80 V at 4 °C. After electrophoresis the gel was dried, exposed to a phosphorimager screen, and analyzed using Image Quant software (Molecular Dynamics). Specificity of tRNA^{Val} recognition by ValRS was monitored with ^{32}P-labeled *E. coli* tRNA^{Cys}, prepared as previously described (*35*). *E. coli* CysRS, which served as a control in these experiments, was prepared according to ref *35*.

RESULTS

Hydrolytic Editing by E. coli ValRS. Proofreading by aminoacyl-tRNA synthetases involves hydrolysis of the enzyme-bound misacylated aminoacyl-AMP either directly (pretransfer) or by rapid deacylation of mischarged tRNA following transitory formation of aminoacyl-tRNA (post-transfer). A tRNA-dependent hydrolysis of ATP, to yield AMP plus PP_i, in the presence of noncognate amino acids, is diagnostic of the overall editing reaction (pre- plus posttransfer). Several amino acids promote editing by *E. coli* ValRS. For example, threonine but not the cognate amino acid valine stimulates ATP hydrolysis (Table 1). In addition, alanine, serine, and cysteine, as well as the nonprotein amino acids α-aminobutyrate and, to a lesser degree, norvaline, promote the editing reaction (Table 1). A number of other amino acids fail to promote editing (data not shown; see legend to Table 1). These results confirm and extend those of previous studies (*3*, *36*, *37*).

As noted earlier (*2*, *3*), the editing reaction (ATP hydrolysis) catalyzed by ValRS requires the presence of tRNA^{Val} (Tables 1 and 2). Unlike wild-type tRNA^{Val}, valine tRNAs having the 3′-terminal adenine replaced with any other base fail to stimulate the editing activity of ValRS (Table 2) even though these modified tRNAs are readily aminoacylated with valine (*26*, *27*, *33*; see Figure 1A). Evidently, the editing site of ValRS recognizes adenosine at the 3′-end of tRNA but fails to respond to U, C, or G at the 3′-end. Substitution of other bases in the 3′-CCA sequence of tRNA^{Val} does not affect the ability of the tRNA to stimulate editing by ValRS. The only exception is the G75 mutant, whose activity is reduced to 4% that of the wild-type tRNA (K. Tardif and J. Horowitz, unpublished results).

Table 1: Amino Acid Stimulation of Editing by *E. coli* ValRS[a]

amino acid	rate of ATP hydrolysis (pmol/min)[b]	relative rate of ATP hydrolysis
threonine	357	(1.0)
valine	−17.5	−0.05
alanine	46.6	0.13
serine	12.7	0.04
cysteine	102	0.29
α-aminobutyrate	307	0.86
norvaline	7.3	0.02
no amino acid	−13.9	−0.04

[a] ATP hydrolysis was monitored at ValRS, tRNA^{Val}, and amino acid concentrations of 2 μM, 1 μM, and 37 mM, respectively. Amino acids and amino acid analogues tested that failed to stimulate editing include isoleucine, leucine, glycine, aspartic acid, asparagine, glutamate, glutamine, lysine, phenylalanine, tyrosine, tryptophan, histidine, proline, allothreonine, D-valine, D-threonine, D-alanine, D-serine, and D-cysteine. [b] Negative values are the result of small variations in the background rate of ATP hydrolysis.

Table 2: Transfer RNA-Dependent Stimulation of Editing by *E. coli* ValRS[a]

tRNA^{Val} variant	rate of ATP hydrolysis (pmol/min)[b]	relative rate of ATP hydrolysis
wild type (A76)	845	(1.0)
U76	−25	−0.03
C76	−88	−0.10
G76	−29	−0.03
no tRNA	−51	−0.06

[a] ATP hydrolysis was monitored in the presence of 37 mM threonine at ValRS and tRNA concentrations of 2 and 1 μM, respectively. [b] Negative values are the result of small variations in the background rate of ATP hydrolysis.

Misacylation of 3′-Terminal Variants of E. coli tRNA^{Val}. The amino acids activated by ValRS, with the exception of the cognate valine, are not stably transferred to wild-type tRNA^{Val} (Figure 1B, Table 3). However, 3′-end variants of tRNA^{Val} terminating in 3′-U or 3′-C, which accept valine (*26*, *27*, *33*; Figure 1A), are fully and stably mischarged with threonine (Figure 1B, Table 3). No significant mischarging of the tRNA^{Val} mutant terminating in 3′-G is observed (Figure 1B, Table 3), although this tRNA can be aminoacylated with valine (Figure 1A).

Misacylation of 3′-end mutants of tRNA^{Val} is not limited to threonine. The U76 and C76 variants of tRNA^{Val} also accept alanine, serine, cysteine, and isoleucine at levels ranging from 10% to 50% those of the aminoacylation with valine (Figure 1C and Table 3). Again, tRNA^{Val} terminating in 3′-G is misacylated poorly with these amino acids (Table 3). Clearly, the identity of the base at the 3′-end of tRNA^{Val} plays an important role in enabling ValRS to effectively differentiate between cognate and noncognate amino acids. Several other amino acids, leucine, methionine, glycine, glutamic acid, lysine, tyrosine, and phenylalanine, are not stably charged onto these 3′-end variants of tRNA^{Val} (Table 3).

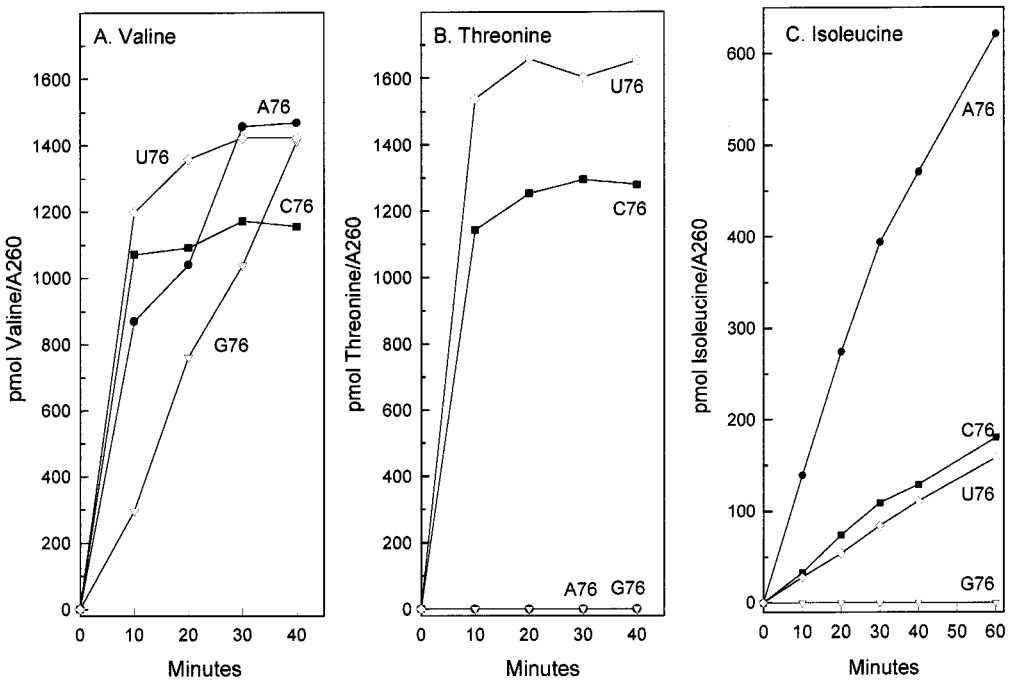

FIGURE 1: Time course of aminoacylation of tRNA^{Val} variants with (A) valine, (B) threonine, and (C) isoleucine. ValRS concentration is 0.2 μM.

Table 3: Level of Misacylation of 3′-Terminal Variants of *E. coli* tRNA^{Val} with Noncognate Amino Acids[a]

	pmol of amino acid/A_{260}			
amino acid	A76 (wild type) tRNA	U76 tRNA	C76 tRNA	G76 tRNA
valine	1294	1406	1211	1372
threonine	0	1628	1312	ND[b] (36)[c]
alanine	0	481	604	ND[b] (1.6)[c]
serine	0	138	248	0
cysteine	0	365	387	51
isoleucine	546	135	155	0
leucine	no detectable misacylation			
methionine	no detectable misacylation			
glycine	no detectable misacylation			
glutamic acid	no detectable misacylation			
lysine	no detectable misacylation			
tyrosine	no detectable misacylation			
phenylalanine	no detectable misacylation			

[a] Aminoacylation levels were determined over a 60 min period at a ValRS concentration of 0.2 μM. [b] ND, no misacylation. [c] Aminoacylation level at 0.046 μM ValRS.

Misacylation of E. coli tRNA^{Val} with Isoleucine. We find that ValRS stably misacylates wild-type tRNA^{Val} with isoleucine (Table 3). This result is surprising because the "double-sieve" editing mechanism (*2*) proposes that amino acids such as isoleucine, which are larger than valine, should be rejected by the activation site of ValRS by steric exclusion. To unambiguously characterize the amino acid bound to the tRNA, wild-type tRNA^{Val} was aminoacylated separately with [^{14}C]isoleucine and [^{14}C]valine. Labeled aminoacyl-tRNAs were isolated and stripped of amino acid under alkaline conditions, and the amino acid was identified by thin-layer chromatography. The amino acids recovered from isoleucyl-tRNA^{Val} and valyl-tRNA^{Val} migrated on TLC plates identically with isoleucine and valine standards, respectively (Figure 2), demonstrating that tRNA^{Val} had been mischarged with isoleucine.

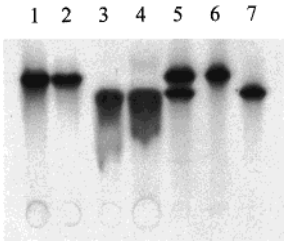

FIGURE 2: Identification of isoleucine aminoacylated to tRNA^{Val}. Amino acids stripped from tRNA^{Val} charged by ValRS with ^{14}C-labeled valine or isoleucine were identified by TLC (see Materials and Methods). Lanes: 1, a mixture of [^{14}C]isoleucine plus tRNA^{Val}; 2, amino acid stripped from tRNA^{Val} aminoacylated with [^{14}C]-isoleucine; 3, amino acid stripped from tRNA^{Val} aminoacylated with [^{14}C]valine; 4, a mixture of [^{14}C]valine and tRNA^{Val}; 5, a mixture of [^{14}C]isoleucine and [^{14}C]valine; 6, [^{14}C]isoleucine; 7, [^{14}C]valine.

Table 4: Kinetic Parameters for Aminoacylation of tRNA^{Val} with Valine and Isoleucine by *E. coli* ValRS

amino acid	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	V_{max}/K_m	relative V_{max}/K_m
valine	39.5	6.41	0.16	(1)
isoleucine	19×10^2	0.023	1.2×10^{-5}	7.5×10^{-5}

The K_m and V_{max} for isoleucine in the ValRS-catalyzed aminoacylation reaction are 48-fold higher and 280-fold lower, respectively, than the corresponding values for valine (Table 4). This translates into a >4 orders of magnitude decrease in the catalytic efficiency of aminoacylation with isoleucine compared to that with valine and accounts for the lack of misacylation of tRNA^{Val} with isoleucine in vivo.

Posttransfer Editing by Valyl-tRNA Synthetase. The ability of ValRS to edit mischarged tRNAs (posttransfer editing) was examined by measuring the enzyme-catalyzed deacylation of misacylated tRNAs. Threonyl-tRNA^{Val} with pyrimidines at the 3′ position (C76 or U76) is not deacylated by ValRS (Figure 3A). Threonylated tRNA^{Val}(G76) is slowly

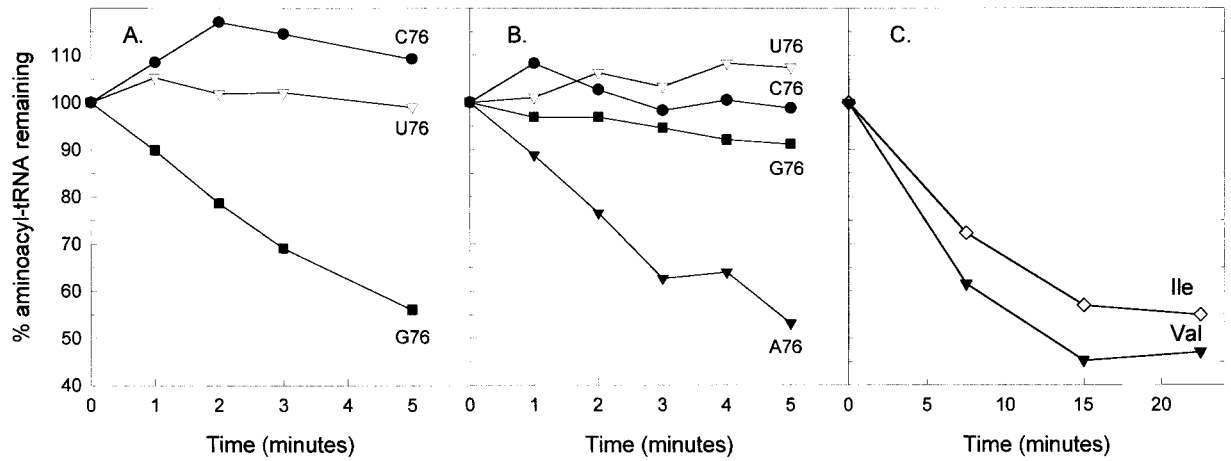

FIGURE 3: Valyl-tRNA synthetase catalyzed deacylation of aminoacylated tRNAs. (A) Deacylation of $tRNA^{Val}$ variants charged with threonine: (●) $tRNA^{Val}$(C76); (▽) $tRNA^{Val}$(U76); (■) $tRNA^{Val}$(G76). (B) Deacylation of $tRNA^{Val}$ variants charged with valine: (●) $tRNA^{Val}$(C76); (▽) $tRNA^{Val}$(U76); (■) $tRNA^{Val}$(G76); (▼) $tRNA^{Val}$(A76) (wild type). (C) Deacylation of isoleucyl-$tRNA^{Val}$ (◇) and valyl-$tRNA^{Val}$ (▼). Aminoacyl-tRNA concentration was 2 μM, and ValRS concentration was 30 nM in (A) and 1.8 μM in (B) and (C). All results are corrected for the spontaneous hydrolysis of aminoacyl-tRNA in the absence of enzyme.

deacylated, at a rate of 0.4 pmol/min (Figure 3A). We were unable to prepare threonylated wild-type $tRNA^{Val}$ for study in these experiments because the proofreading reaction of ValRS (pre- and/or posttransfer) is too rapid in the presence of wild-type $tRNA^{Val}$. Evidently, a purine at the 3′-terminal position of $tRNA^{Val}$ is necessary for ValRS to catalyze the deacylation of threonylated $tRNA^{Val}$ (posttransfer editing).

Aminoacyl-tRNA synthetases are weak deacylases of their cognate aminoacyl-tRNAs, e.g., yeast ValRS (*38*) and *E. coli* IleRS (*39*). *E. coli* ValRS, at reasonably high concentrations (1.8 μM) relative to that of the tRNA substrate (2 μM), slowly deacylates wild-type (A76) $tRNA^{Val}$ charged with valine (Figure 3B). However, ValRS does not deacylate, or only very slowly deacylates, valyl-$tRNA^{Val}$(G76) (Figure 3B), even though it does hydrolyze the ester bond of this tRNA mischarged with threonine (Figure 3A). As expected, because the editing site of ValRS does not recognize pyrimidines, valyl-$tRNA^{Val}$ terminating in 3′-U or 3′-C is not deacylated (Figure 3B). ValRS deacylates isoleucine misacylated to wild-type $tRNA^{Val}$ at a rate similar to that of the synthetase-catalyzed deacylation of valyl-$tRNA^{Val}$ (Figure 3C).

Inhibition of Aminoacylation by tRNAs. The possibility that ValRS fails to deacylate threonyl-$tRNA^{Val}$(U76) and threonyl-$tRNA^{Val}$(C76) because these misacylated tRNAs do not bind to the enzyme can be ruled out because they are effective inhibitors of the aminoacylation of wild-type $tRNA^{Val}$ with valine. This is shown in Figure 4 for threonyl-$tRNA^{Val}$(C76) and clearly indicates that mischarged tRNAs interact with ValRS. Surprisingly, mischarged tRNAs act as mixed noncompetitive inhibitors of the aminoacylation reaction (Figure 4) with $K_I = 8.5$ μM and $K_I' = 94$ μM (Table 5). Noncompetitive inhibition of aminoacylation of $tRNA^{Val}$ is also observed with 3′-end-truncated tRNAs (*40*): $tRNA^{Val}$ missing the terminal −CCA sequence [$tRNA^{Val}$(−CCA)] and that lacking the 3′-ACCA end [$tRNA^{Val}$(−ACCA)] (Table 5). The kinetic parameters show that K_I', the dissociation constant for the enzyme−substrate−inhibitor complex is 5−11 times greater than K_I, the dissociation constant of the enzyme−inhibitor complex (Table 5), indicating that free ValRS has a greater affinity for the inhibitors than does the enzyme when it is associated with a tRNA substrate molecule.

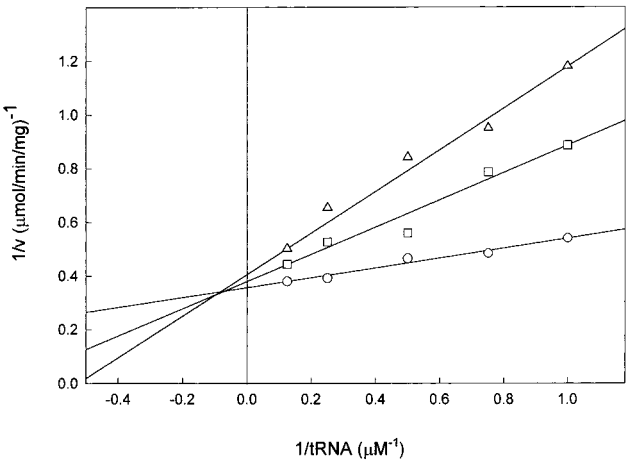

FIGURE 4: Lineweaver−Burk plot of the inhibition of valylation of wild-type $tRNA^{Val}$ by threonyl-$tRNA^{Val}$(C76): no inhibitor (△); 10 μM threonyl-$tRNA^{Val}$(C76) (□); 20 μM threonyl-$tRNA^{Val}$(C76) (○). Experiments were carried out at pH 6.5 to minimize spontaneous hydrolysis of the aminoacyl bond.

Table 5: Kinetic Parameters for Inhibition of $tRNA^{Val}$ Aminoacylation by 3′-End-Modified tRNAs[a]

inhibitor	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	K_I (μM)	K_I' (μM)
Thr-C76 $tRNA^{Val}$	1.0	4.8	8.5	94.1
$tRNA^{Val}$ (−ACCA)	2.9	10.7	20.4	141
$tRNA^{Val}$ (−CCA)	3.4	12.2	28.8	148

[a] K_I and K_I' were determined from the regression analysis using a statistical MINITAB program. The kinetic data are averages of two experiments.

A Second tRNA Binding Site on Valyl-tRNA Synthetase. Because noncompetitive inhibition of aminoacylation by misacylated $tRNA^{Val}$(C76) and the truncated tRNAs suggests a second tRNA binding site on ValRS in addition to the site at which the tRNA is aminoacylated, we analyzed the stoichiometry of the tRNA−ValRS complex by the method of continuous variations (*41*, *42*), taking advantage of the shift in electrophoretic mobility resulting from the binding of tRNA to enzyme (gel retardation). Varying concentrations of ^{32}P-labeled $tRNA^{Val}$ and ValRS (His tagged) were incubated for 15 min at room temperature, at which time

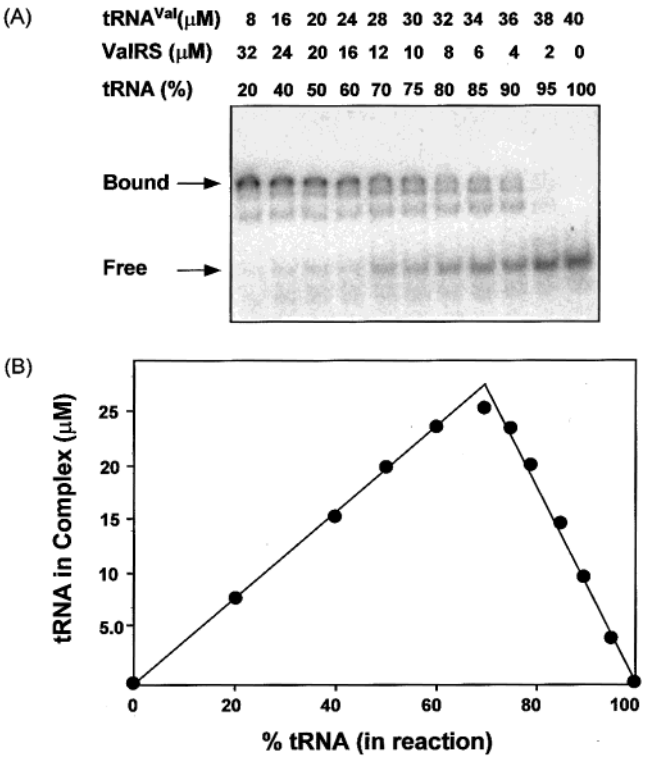

FIGURE 5: Stoichiometry of the binding interaction between *E. coli* $tRNA^{Val}$ (T7 transcript) and *E. coli* ValRS. ^{32}P-Labeled $tRNA^{Val}$ and ValRS were mixed at the indicated ratios and incubated at room temperature for 15 min. The equilibrium was fixed by addition of 0.2% glutaraldehyde, and the reaction was loaded on a native 8% polyacrylamide gel. (A) Gel-mobility shift assay of ValRS binding to radiolabeled $tRNA^{Val}$. (B) Analysis of the concentration of tRNA in the complex as a function of the percentage of tRNA in the reaction shows a peak at approximately 70% tRNA, corresponding to a 2:1 tRNA−enzyme complex.

the binding equilibrium was fixed by addition of 0.2% glutaraldehyde, which inactivates the enzyme. The concentrations of ValRS and ^{32}P-labeled $tRNA^{Val}$ varied from 0 to 40 μM, but the concentration of tRNA plus ValRS was maintained constant at 40 μM. Enzyme-bound tRNA and free tRNA were separated by native gel electrophoresis, and the radioactive tRNA was visualized and quantitated by phosphorimaging. Two band shifts are observed (Figure 5A). The major band, labeled bound tRNA in Figure 5A, corresponds to a 2:1 tRNA−protein complex. This is demonstrated in Figure 5B, which shows a plot of the concentration of tRNA in the major shifted band as a function of the mole percent of tRNA in the reaction. In such a binding experiment, the peak of complex formation represents the stoichiometry of the complex (*41*, *42*). Reactions at points other than the peak represent conditions under which one component is limiting relative to the other. Results in Figure 5B show that the peak of complex formation occurs at the point where tRNA represents approximately 70% of the interacting components, indicating a stoichiometry of two tRNAs per ValRS. The minor shifted band, retarded somewhat less than the major band in Figure 5B, may represent an intermediate complex having a single tRNA molecule bound per ValRS. To examine the specificity of $tRNA^{Val}$ binding to ValRS, the ability of the enzyme to associate with noncognate *E. coli* $tRNA^{Cys}$ was monitored by a gel-mobility-shift experiment. ValRS does not bind $tRNA^{Cys}$ under conditions of the experiment, nor does CysRS (which binds $tRNA^{Cys}$) interact with $tRNA^{Val}$ (results not shown).

DISCUSSION

E. coli valyl-tRNA synthetase misactivates several noncognate amino acids, most notably threonine, but also alanine, serine, cysteine, α-aminobutyrate, and norvaline (Table 1). Because of the hydrolytic editing reaction catalyzed by the enzyme, wild-type $tRNA^{Val}$ is not stably aminoacylated with these amino acids (Table 3, Figure 1B). This proofreading activity is strongly stimulated by the cognate wild-type $tRNA^{Val}$ but not by $tRNA^{Val}$ mutants having the 3′-adenosine replaced by any other nucleoside (Table 2). As a result, valine tRNAs terminating in C76 or U76, unlike wild-type tRNA (A76), are stably misacylated with threonine, alanine, serine, cysteine, and isoleucine (Figure 1, Table 3). These 3′-end mutants of $tRNA^{Val}$ had previously been shown to readily accept valine (*26*, *27*, *33*; Figure 1A). Little or no misacylation of the mutant $tRNA^{Val}$ terminating in 3′-G is observed (Figure 1, Table 3). Evidently, the identity of the base at the 3′-end of the tRNA is important for the editing response.

Misacylated valine tRNAs that terminate in 3′-pyrimidines are not edited by ValRS; the enzyme does not hydrolyze the ester bond of threonyl-$tRNA^{Val}$(C76 and U76) (Figure 3A). However, threonyl-$tRNA^{Val}$ terminating in 3′-G is deacylated at a measurable rate (Figure 3A), and wild-type (3′-A) threonyl-$tRNA^{Val}$ is deacylated too rapidly to be isolated. Clearly, a purine, either A or G, is required at the 3′-terminus of misacylated $tRNA^{Val}$ for ValRS to catalyze posttransfer editing.

The recently solved crystal structure of *Thermus thermophilus* ValRS complexed with $tRNA^{Val}$ and a Val-AMP analogue at 2.9 Å resolution (*20*) shows that the editing domain of the enzyme specifically recognizes the 3′-terminal adenosine of the tRNA. The purine ring of A76 is sandwiched between the side chains of Phe^{264} and Leu^{269} by van der Waals contacts, and the N1 atom and 6-NH_2 group of A76 form H-bonds with the α-NH and α-CO groups, respectively, of Glu^{261}. Amino acids participating in the base-specific recognition of A76 are highly conserved among ValRS (*20*). Such an extensive network of hydrophobic interactions and H-bonds emphasizes the importance of adenosine at the 3′-end of $tRNA^{Val}$ and explains why substitution of A76 has a major perturbing effect on the editing reaction. Because the purine ring of 3′-terminal guanosine can also stack between the side chains of hydrophobic amino acids, the editing site of ValRS can recognize and deacylate misacylated threonyl-$tRNA^{Val}$(G76) (Figure 3A). Absence of the 6-NH_2 group and protonation of the N1 of guanosine could explain the slower deacylation rate. Pyrimidines stack poorly, and this may prevent proper recognition of the 3′-end of the 3′-U and 3′-C variants of $tRNA^{Val}$ at the editing site of ValRS, interfering with the editing reaction.

Our finding that ValRS stably aminoacylates wild-type $tRNA^{Val}$ and 3′-end variants of $tRNA^{Val}$ with isoleucine (Table 3 and Figure 1C) is unexpected. Fersht and Dingwall (*36*) concluded that an apparent misactivation of isoleucine by ValRS is due to contamination of commercial isoleucine preparations with valine. The results in Figure 2, however,

clearly identify the amino acid charged to $tRNA^{Val}$ as isoleucine. Fersht and colleagues have proposed a double-sieve mechanism to account for the specificity of the editing reaction (*2*, *36*, *37*). In this scheme the first (coarse) sieve rejects amino acids larger than the cognate. Smaller or isosteric amino acids are activated and then hydrolyzed at a separate site, the second (fine) sieve, which excludes the cognate amino acid on the basis of size or lack of essential functional groups. Isoleucine, being larger than valine, should be excluded from the aminoacylation site of ValRS. It is evidently not completely rejected and is, like threonine, alanine, serine, and cysteine, misactivated by the enzyme. Recently, Hountondji et al., in studies using reactive amino acid analogues, reported that misactivation of isoleucine (and other noncognate amino acids) by *E. coli* ValRS occurs at a subsite of the aminoacylation site close to but separate from that for activation of the cognate valine (*43*). Unlike threonine, alanine, serine, and cysteine, isoleucine (like the cognate valine) is not readily accommodated at the editing site of ValRS and, therefore, becomes stably aminoacylated onto $tRNA^{Val}$ (Figure 1C). The observation that isoleucyl-$tRNA^{Val}$ is only slowly deacylated by ValRS, at about the same rate as the cognate valyl-$tRNA^{Val}$ (Figure 3C), is consistent with this view. The large hydrophobic side chains of both amino acids are not readily accepted at the editing site because of their size and because they lack proper hydrogen-bonding groups to interact with the hydrophilic amino acids that line the editing pocket of the enzyme (*20*).

Misacylation of $tRNA^{Val}$ with isoleucine is so inefficient that no in vivo editing is required. Valine is a much better substrate for ValRS than isoleucine (Table 4), and the intracellular concentration of valine in *E. coli* is more than five times that of isoleucine (*44*). As a result, valine readily out competes isoleucine in the aminoacylation reaction in vivo.

Although ValRS is unable to deacylate misacylated 3′-end mutants of $tRNA^{Val}$, the synthetase does interact with these tRNAs as shown by inhibition of valine aminoacylation of wild-type $tRNA^{Val}$ by mischarged mutant tRNAs (Figure 4 and Table 5). Truncated valine tRNAs, $tRNA^{Val}$(−CCA) and $tRNA^{Val}$(−ACCA), which lack the 3′-terminal CCA or ACCA sequence and are not aminoacylated by ValRS, are also inhibitors of $tRNA^{Val}$ aminoacylation (Table 5). In both cases, the tRNAs act as mixed noncompetitive inhibitors of the reaction (Figure 4, Table 5). Yeast IleRS, a class I synthetase closely related to ValRS, is also noncompetitively inhibited by cognate tRNAs having modified 3′-terminal ends (*45*). A second tRNA binding site on the monomeric enzyme, in addition to the site at which the tRNA is aminoacylated, most readily explains these results. In this connection it may be noted that *E. coli* ValRS has two binding sites for the small substrate valine (*46*).

The mobility-shift experiment (Figure 5) provides direct evidence for the binding of more than one $tRNA^{Val}$ molecule to *E. coli* ValRS, a monomeric enzyme. How ValRS simultaneously recognizes two molecules of $tRNA^{Val}$ is not clear. It is known that the anticodon is the strongest synthetase recognition determinant of the tRNA (*40*, *47*, *48*). Valine tRNA with nucleotide substitutions for A35 or C36, in the anticodon, fails to interact with ValRS, as shown by its inability to inhibit aminoacylation of wild-type $tRNA^{Val}$ (*40*). For the second tRNA to be recognized and bound by ValRS, the enzyme must release the anticodon of the first tRNA. This implies a conformational change in the anticodon-binding domain of ValRS, or of the tRNA, during the exchange between the two tRNAs, to allow the enzyme to disengage the anticodon of the first tRNA. The acceptor end of one tRNA may be positioned in the aminoacylation site while that of the second is in the editing site. Occupancy of the editing site may directly interfere with the function of the aminoacylation site, or aminoacylation of the first tRNA may be indirectly affected due to dislodging of its anticodon from the enzyme. Further biochemical analysis of the enzyme−tRNA interaction, combined with additional modeling studies of the ValRS−tRNA complex, should provide further insight into the proposed model.

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