Crucial Role of Conserved Lysine 277 in the Fidelity of tRNA Aminoacylation by *Escherichia coli* Valyl-tRNA Synthetase†

Codjo Hountondji,*,‡ Christine Lazennec,‡ Christian Beauvallet,§ Philippe Dessen,‡,|| Jean-Claude Pernollet,§ Pierre Plateau,‡ and Sylvain Blanquet‡

Laboratoire de Biochimie, CNRS UMR 7654, Ecole Polytechnique, 91128 Palaiseau Cedex, France, Institut National de la Recherche Agronomique (INRA), Unité de Recherches en Biochimie et Structure des Protéines, Domaine de Vilvert, 78352 Jouy-en-Josas Cedex, France, and Laboratoire de Génétique Oncologique, CNRS UMR 1599, Institut Gustave Roussy PR1, Rue C. Desmoulins, 94805 Villejuif Cedex, France

Received August 2, 2002; Revised Manuscript Received October 17, 2002

ABSTRACT: Valyl-tRNA synthetase (ValRS) from *Escherichia coli* undergoes covalent valylation by a donor valyl adenylate synthesized by the enzyme itself. ValRS could also be modified, although to a lesser extent, by the noncognate isosteric substrate L-threonine from a donor threonyl adenylate synthesized by the synthetase itself, or by the nonsubstrate methionine from methionyl adenylate produced by catalytic amounts of methionyl-tRNA synthetase. MALDI mass spectrometry analysis designated lysines 154, 162, 170, 533, 554, 593, 894, 930, and 940 of ValRS as the target residues for the attachment of valine. Following autothreonylation, lysines 162, 170, 178, 277, 291, 554, 580, 593, 861, 894, and 930 were found to be modified. Finally, L-Met-labeled residues were lysines 118, 162, 170, 178, 277, and 938. Alignment of the available ValRS amino acid sequences showed that lysines 277 and 554 are strictly conserved (with the exception concerning replacement of Lys-277 with a methionine or a tyrosine in archaebacteria), suggesting that these residues might be functionally significant. Indeed, lysine 554 of ValRS is the first lysine of the Lys-Met-Ser-Lys-Ser signature of the catalytic site of class I aminoacyl-tRNA synthetases. Lys-277 which is labeled by L-threonine or L-methionine, and not by L-valine, is located at or near the editing site, in the three-dimensional structure of ValRS. The role of lysine 277 was evaluated by site-directed mutagenesis. The Lys277Ala mutant (K277A) exhibited a posttransfer Thr-tRNA^{Val} editing rate that was significantly lower than that observed for the wild-type enzyme. In addition, the K277A substitution altered amino acid discrimination in the editing site, resulting in hydrolysis of the correctly charged cognate Val-tRNA^{Val}. Finally, significant amounts of mischarged Thr-tRNA^{Val} were produced by the K277A mutant, and not by wild-type ValRS. Altogether, our results designate Lys-277 as a likely candidate for nucleophilic attack of misacylated tRNA in the editing site of ValRS.

An essential factor in the accuracy of protein biosynthesis is the editing or proofreading activity of certain aminoacyl-tRNA synthetases whereby the products of misactivated amino acids are removed somehow by hydrolysis. For example, IleRS is remarkably accurate in that it causes misincorporation of the closely related valine for isoleucine into the proteins ovalbumin and globin in only 1 of 3000 positions (*1*). Yet, *Escherichia coli* IleRS activates L-valine at a relative frequency of ~0.5% (*2*). Similarly, while ValRS activates the isosteric noncognate substrate L-threonine and can form a 1:1 complex with threonyl adenylate, it does not catalyze the net formation of Thr-tRNA^{Val} (*3*).

The editing reaction is composed of two separate pathways qualified as pretransfer and posttransfer steps (*3*, *4*). In pretransfer editing, the ATP-activated noncognate amino acid is cleared before being transferred to the 3′-end of cognate tRNA (aminoacyl-AMP hydrolysis), while in posttransfer editing, the noncognate amino acid is cleared after being esterified to the tRNA (aminoacyl-tRNA hydrolysis). Accordingly, the aminoacyl-tRNA synthetases concerned with editing are supposed to exhibit two catalytic sites, one for aminoacylation through the activation of the specific amino acid and one for editing of misactivated or misesterified noncognate substrates (*3*, *4*).

In the class I aminoacyl-tRNA synthetases, the Lys-Met-Ser-Lys-Ser consensus sequence (*5*) represents the signature of the aminoacylating site, while the so-called connecting peptide (CP1) was hypothesized to contain a putative editing active site (*6*). Mutational analyses of the CP1 domain that is highly conserved across bacteria, archaea, and eukarya were the subject of numerous investigations in the ValRS (*7*, *8*), IleRS (*9*−*11*), and LeuRS (*12*, *13*) families. Mutations in the CP1 domain of IleRS and ValRS have produced enzyme variants that misacylated cognate tRNAs with noncognate amino acids and/or showed an inability to liberate noncognate amino acids that had been incorrectly charged

† This work was supported by grants from CNRS and the Association pour la Recherche sur le Cancer (ARC).

* To whom correspondence should be addressed: Laboratoire de Biochimie, CNRS UMR 7654, Ecole Polytechnique, 91128 Palaiseau Cedex, France. Telephone: (33) (0)1.69.33.48.84. Fax: (33) (0)1.69.33.30.13. E-mail: codjo@botrytis.polytechnique.fr.

‡ Ecole Polytechnique.

§ Unité de Recherches en Biochimie et Structure des Protéines.

|| Institut Gustave Roussy PR1.

10.1021/bi0205101 CCC: $22.00
Published on Web 11/16/2002

on cognate tRNAs. Mutants of *E. coli* ValRS that incorrectly charge $tRNA^{Val}$ with cysteine were also selected after random mutagenesis of the whole chromosome. All mutations that were obtained (T222P, R223H, D230N, V276A, and K277Q) were clustered in the putative editing site of the synthetase. One variant (T222P) was partially purified and could be shown to misacylate $tRNA^{Val}$ in vitro with either Thr or Cys (*8*). However, the activity in Val-$tRNA^{Val}$ production of the mutant enzyme remained identical to that of wild-type ValRS. The studies described above demonstrate the crucial role of CP1 in the formation of an editing site.

The molecular mechanisms of editing are not yet completely elucidated. For example, a hydrolytic mechanism analogous to the charge relay system of the serine proteases has been hypothesized in the case of *Thermus thermophilus* IleRS (*9*). In the case of the ValRS and LeuRS families, a mechanism has not yet been established.

Functional identification of the residues involved in the editing activity is a prerequisite for the elucidation of the molecular mechanism of edition. The affinity labeling approach may be useful for this purpose.

In this study, we have taken advantage of autoaminoacylation of ValRS to search for amino acid residues belonging to the binding sites of the cognate substrate L-valine or of the noncognate isosteric substrate L-threonine. In our approach, both the catalytic and editing sites were supposed to be targets of the acylation reaction directed by the noncognate adenylate, while only the catalytic site should react with the cognate valyl adenylate.

EXPERIMENTAL PROCEDURES

Materials. Monomeric truncated MetRS from *Bacillus stearothermophilus* (MS534) was purified from *E. coli* cells transformed by the plasmid pUC12MS534 (*14*). Its molar concentration was calculated from its absorbency at 280 nm and from the molecular ratio of 62K and the optical extinction coefficient of 1.47 units mg^{-1} cm^2.

Inorganic pyrophosphatase from yeast was purchased from Roche as a suspension in ammonium sulfate at a concentration of 1 mg/mL. Prior to use, the enzyme was dialyzed against 0.1 M Hepes-Na buffer (pH 8.0) to eliminate ammonium sulfate. *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes)[1] was from Sigma. Sequence grade TosPheCH_2Cl-treated trypsin was from Roche. [^{14}C]-L-Methionine, [^{14}C]-L-threonine, and [^{14}C]-L-valine were from NEN and exhibited specific radioactivities of 57.9, 214, and 283 mCi/mmol, respectively. Matrix solutions and the peptide standard for MALDI-MS analyses were from Interchim (Monluçon, France). Oligonucleotides were purchased from Genome Express (Montreuil, France). Restriction and modification enzymes were from Roche.

Covalent Attachment of Amino Acids to ValRS. ValRS (2 μM) was incubated at 37 °C in 0.1 M Hepes-Na buffer (pH 8.0) containing 2 mM MgATP, 8 mM free $MgCl_2$, 5 μg/mL pyrophosphatase, and 100 μM [^{14}C]-L-valine (63.9 mCi/mmol). For the threonylation reaction, the composition of the incubation mixture was the same as above, except that [^{14}C]-L-valine was replaced with [^{14}C]-L-threonine (400 μM, 43.5 mCi/mmol) and the ValRS concentration was set to 10 μM. For the methionylation of ValRS, the donor methionyl adenylate was synthesized by catalytic amounts (final concentration of 50−100 nM) of *B. stearothermophilus* MetRS added to the incubation mixture, while [^{14}C]-L-valine was replaced with 300 μM [^{14}C]-L-methionine (57.9 mCi/mmol). At different times, 20−50 μL aliquots were withdrawn and added to 3 mL of 5% trichloroacetic acid (TCA). After addition of 200 μg of bovine serum albumin or of unfractionated yeast RNA, the samples were allowed to stand for 1 h in an ice bath before filtration on glass fiber filters and counting of the filters in a liquid scintillation counter. In parallel, when necessary, 3−5 μL aliquots of the incubation mixtures were withdrawn, diluted at least 100-fold with 20 mM Tris-HCl buffer (pH 7.8) containing 200 μg/mL bovine serum albumin, 10 mM 2-mercaptoethanol, and 0.1 mM EDTA, and assayed for activity in the valine-dependent [^{32}P]PP_i−ATP exchange and $tRNA^{Val}$ aminoacylation reactions (*15*, *16*).

Preparation of Labeled Peptides of ValRS. ValRS (10 μM in 5 mL) was incubated at 37 °C in 0.1 M Hepes-Na buffer (pH 8.0) containing 2 mM ATP, 10 mM $MgCl_2$, 5 μg/mL pyrophosphatase, and 100 μM [^{14}C]-L-Val (63.9 mCi/mmol) or 400 μM [^{14}C]-L-Thr (43.5 mCi/mmol). For the preparation of methionylated ValRS, the composition of the incubation mixture was the same as above, except that the donor methionyl adenylate was synthesized from 300 μM [^{14}C]-L-Met (57.9 mCi/mmol) by 100 nM *B. stearothermophilus* MetRS added to the incubation mixture. After 300 min, when the level of incorporation had reached a plateau value, the sample was extensively dialyzed against 0.1 M ammonium acetate (pH 8.0). The labeled ValRS was digested overnight at 37 °C with TosPheCH_2Cl-treated trypsin at a protease:substrate ratio of 1:20 (w/w). The tryptic digest was applied to an Alltima (Alltech) C18 column (3.2 mm × 150 mm, 5 μm particle size) equilibrated with 0.1% TFA in water. The tryptic peptides were then eluted with linear gradients of acetonitrile in 0.1% TFA. Peaks were detected by recording the absorbance at 215 nm, as well as by radioactivity measurements in a liquid scintillation counter. As a control, an intact ValRS sample (10 μM in 5 mL), incubated alone for 300 min at 37 °C in 0.1 M Hepes-Na buffer (pH 8.0), was dialyzed against 0.1 M ammonium acetate (pH 8.0) and submitted to trypsin digestion and RPLC separation under the same conditions as the labeled synthetase, with monitoring at 215 nm.

Mass Spectrum Analyses. Samples of labeled peptides were prepared by mixing 1 μL of matrix solution of 100 mM α-cyano-4-hydroxycinnamic acid (Hewlett-Packard G2037A) with 1 μL of peptides (5−10 pmol). This mixture was then loaded on the probe tip and vacuum-dried.

MALDI-TOF spectra were obtained on a linear time-of-flight mass spectrometer (Hewlett-Packard G2025). Samples were prepared with a Hewlett-Packard G2024 accessory. From 100 to 200 shots were accumulated for each spectrum acquisition in the positive ion mode. Calibration with external standards was achieved with a mixture of peptide standard (Hewlett-Packard G2052A) in the range of 1000−7000 Da. MALDI mass spectra were also recorded on a Perkin-Elmer

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry. Aminoacyl-tRNA synthetases are abbreviated with the three-letter codes of their specific amino acid followed by RS; amino acid residues are abbreviated with the one- or three-letter code.

Voyager spectrometer equipped with a 337 nm laser, with an acceleration voltage of 20 kV.

Plasmid Construction. To introduce the *E. coli valS* gene into plasmid pTrc99A (from Amersham Pharmacia Biotech), a 324 bp DNA fragment was amplified using the plasmid pXVal17 (*17*) as a template and oligonucleotides GCAACCTGGAAACCATGGAAAAGAC and CAGATTCCGCTTTCCATTCCC as primers. After digestion by *Nco*I and *Hin*dIII restriction enzymes, the amplified fragment was inserted between the corresponding sites of pTrc99A. Then, a 4158 bp *Hin*dIII fragment containing the 3′-end of the *valS* gene was introduced into the *Hin*dIII site of the resulting plasmid, to give pTRC-Val.

A 1420 bp *Bgl*II−*Xho*I fragment of *val*S was cloned into the M13mp19 phage vector. Site-directed mutagenesis was then performed on the resulting vector, as previously described (*18*). The mutated fragment was reinserted into pTRC-Val, to give pTRC-ValK277A. The entire sequence of the mutant *valS* gene was verified by DNA sequencing (*19*).

To add a His_6 tag to the C-termini of wild-type and mutant ValRS, a 757 bp DNA fragment was amplified using the plasmid pTRC-ValK277A as a template and oligonucleotides CACCTGGAACCAGTTCTGTGACTGG and CGCTCGCCGGCTTAGTGATGGTGATGGTGATGCAGCGCGGCGATAACAGCCTGCTG as primers. After digestion by *Xho*I and *Nae*I restriction enzymes, the amplified fragment was introduced into the corresponding sites of plasmids pTRC-Val and pTRC-ValK277A, to give pTRC-Val-His_6 and pTRC-ValK277A-His_6, respectively. Proper constructions were verified by DNA sequencing (*19*).

Protein Expression and Purification. Native ValRS was purified from JM101Tr cells harboring the pXVal17 plasmid (*17*). Cells were grown in 1 L of 2×TY medium containing 50 μg/mL ampicillin. Crude extract preparation, nucleic acid precipitation of nucleic acids, ammonium sulfate precipitation of proteins, and chromatographies on Superose 6 and Q-HiLoad columns were performed as described previously for the purification of histidyl-tRNA synthetase (*20*).

The His_6-tagged wild-type and mutant ValRSs were purified from JM101Tr cells harboring the plasmid pTRC-Val-His_6 and pTRC-ValK277A-His_6, respectively. Cells were grown in 2×TY medium containing 50 μg/mL ampicillin. The volume of the culture was 1 L in the case of the wild-type ValRS and 5 L in the case of the mutant enzyme. When the optical density of the cultures reached 1 at 650 nm, IPTG was added at a final concentration of 0.1 mM and growth was continued for 3 h. Crude extract preparation, nucleic acid precipitation of nucleic acids, and ammonium sulfate precipitation of proteins were performed as described previously for the purification of lysyl-tRNA synthetase (*21*). Then, the protein pellet was dissolved in 10−50 mL of 20 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl (buffer A). The resulting solution was applied on a 1 cm × 10 cm column of Ni−NTA agarose (from Qiagen), equilibrated in buffer A. The column was washed with 50 mL of buffer A and eluted with a linear 100 mL gradient from 0 to 50 mM imidazole in buffer A. In the case of the mutant enzyme, two additional chromatographic steps proved to be necessary to obtain the enzyme free from contaminants: a second affinity chromatography on Ni−NTA and an ion exchange chromatography step on a Q-HiLoad column. The recovered homogeneous proteins were stored at −30 °C in 20 mM Tris-HCl buffer (pH 7.6) containing 55% glycerol.

The molar concentration of ValRS was calculated from its absorbency at 280 nm and from a molecular weight of 101K and an optical extinction coefficient of 1.53 units mg^{-1} cm^2.

Finally, MALDI-MS analysis of the fractions of the RP-HPLC profile from a tryptic digest of the K277A mutant enzyme, carried out in comparison with that of wild-type ValRS, enabled confirmation of ∼90% of the sequence of the synthetase. Altogether, the data that were obtained indicated that, apart from the K277A point mutation in the putative editing site, no mutation had occurred in other regions of the variant enzyme.

Preparation of $tRNA^{Val}$ and [^{14}C]Val-$tRNA^{Val}$. *E. coli* $tRNA^{Val1}$ was extracted from strain JM101Tr transformed by plasmid pBStRNAVal1 (*22*). The crude tRNA extract from this strain accepted 700−1000 pmol of valine per A_{260} unit. For the preparation of [^{14}C]Val-$tRNA^{Val1}$, $tRNA^{Val1}$ was fully valylated within 30 min at 25 °C in a solution (1 mL) containing 20 mM Tris-HCl (pH 7.6), 7 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 2 mM ATP, 6 μM $tRNA^{Val1}$, 14 μM [^{14}C]-L-valine (63.9 mCi/mmol), and 1 μM wild-type ValRS. After the mixture had cooled to 0 °C and after addition of 0.1 mL of 3 M sodium acetate buffer (pH 5.0), tRNA was extracted by addition of a saturated solution of phenol. After removal of the phenol by ethanol precipitation, the [^{14}C]Val-$tRNA^{Val1}$ was purified on a Trisacryl GF05M column equilibrated with 20 mM sodium acetate (pH 4.9). Purified [^{14}C]Val-$tRNA^{Val1}$ was stored in 5 mM sodium acetate (pH 4.9). Enzymatic deacylation of [^{14}C]Val-$tRNA^{Val1}$ was assayed at 25 °C, in the presence of 20 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 50 μg/mL BSA, 0.77 μM aminoacyl-tRNA, and 100 nM purified wild-type or mutant ValRS. Reactions (100 μL volume) were quenched by the addition of 5% TCA, and the mixtures were filtered on glass fiber filters (GF/C from Whatman) and quantified by liquid scintillation counting in a Beckman LS 1801 counter. Data were corrected for spontaneous hydrolysis of the aminoacyl bond, followed under the same conditions in the absence of ValRS.

Deacylation of [^{14}C]Thr-$tRNA^{Val1}$. [^{14}C]Thr-$tRNA^{Val1}$ was synthesized in a solution mixture (1 mL) containing 6 μM purified *E. coli* $tRNA^{Val1}$, 80 μg/mL *T. thermophilus* threonyl-tRNA synthetase (a generous gift of D. Kern, IBMC, Strasbourg, France), 10 mM Tris-HCl (pH 9.0), 10 mM $MgSO_4$, 1 mM ATP, 20% methanol, and 46.6 μM [^{14}C]-L-threonine (214.6 mCi/mmol). After a 30 min incubation at 37 °C, [^{14}C]Thr-$tRNA^{Val1}$ was purified on a Trisacryl GF05M column as described above, and stored in 5 mM sodium acetate (pH 4.9). Deacylation of [^{14}C]Thr-$tRNA^{Val1}$ was assayed at 25 °C in the presence of 20 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 50 μg/mL BSA, 0.25 μM aminoacyl-tRNA, and 10 nM purified wild-type ValRS or 20 nM mutant ValRS. Reactions (100 μL volume) were quenched by the addition of 5% TCA, and the mixtures were filtered on glass fiber filters and quantified as described above.

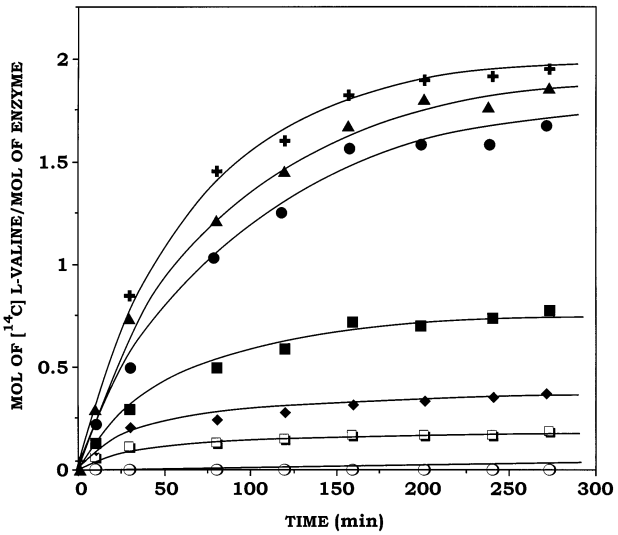

FIGURE 1: Time dependence of valine incorporation by ValRS. The enzyme (2 μM) was incubated at 37 °C in 0.1 M Hepes-Na buffer (pH 8.0) containing 8 mM $MgCl_2$, 2 mM MgATP, 5 μg/mL inorganic pyrophosphatase, and [^{14}C]-L-valine (63.9 mCi/mmol) at final concentrations of 7 (□), 14 (◆), 28 (■), 56 (●), 116 (▲), and 232 μM (+). The number of moles of [^{14}C]-L-Val incorporated per mole of enzyme, as deduced from radioactivity measurements, was plotted as a function of incubation time. Control experiments were carried out with 2 mM ATP without $MgCl_2$, or with 8 mM $MgCl_2$ without ATP (○).

RESULTS AND DISCUSSION

Enzyme-Induced Incorporation of Valyl or Threonyl Residues by ValRS. When ValRS was incubated in the presence of ATP, $MgCl_2$, inorganic pyrophosphatase, and [^{14}C]-L-valine, it underwent autoaminoacylation with valine in a time-dependent manner (Figure 1). [^{14}C]Valine was not incorporated into ValRS in the absence of either ATP or $MgCl_2$, both of which are required for adenylate formation. In addition, the rate and extent of ValRS valylation increased with [^{14}C]valine concentration (Figure 1). Altogether, these results suggest that the valyl adenylate formed by the enzyme itself was the donor of valyl residues for the covalent modification. As already discussed in the case of *E. coli* MetRS (*23*), pyrophosphatase stimulates the autoaminoacylation reaction by displacing the equilibrium of the complexes of the enzyme with substrates toward full aminoacyl adenylate formation.

With 100 μM [^{14}C]-L-valine in the incubation mixture, the stoichiometry of labeling progressively tended to a plateau value, within 3−4 h, corresponding to ∼2 mol of [^{14}C]-L-Val incorporated per mole of ValRS (10 μM). The initial rate of [^{14}C]-L-Val incorporation was 0.029 min^{-1}.

ValRS was also modified by the isosteric noncognate substrate L-threonine. With [^{14}C]-L-Thr (400 μM) instead of L-valine, a maximum of ∼0.06 mol of [^{14}C]-L-Thr was incorporated per mole of ValRS (10 μM). The initial rate of [^{14}C]-L-Thr incorporation into ValRS was 0.0013 min^{-1}. It was verified by thin-layer chromatography on cellulose plates, as well as by RP-HPLC on a C18 column, that the used [^{14}C]-L-Thr sample was free of [^{14}C]-L-Val or of any the other radioactive material. Any attempt to increase the amount of [^{14}C]-L-Thr incorporated by ValRS was unsuccessful.

ValRS could also be covalently modified by methionyl adenylate synthesized by catalytic amounts of MetRS (*23*). Under the standard conditions described in Experimental Procedures, with 100 nM MetRS, and with 300 μM [^{14}C]-L-Met instead of [^{14}C]-L-Val, ValRS (10 μM) incorporated 0.65 mol of [^{14}C]-L-Met per mole of protein. No methionine incorporation occurred if MetRS was omitted.

When ValRS (10 μM) was exposed to threonyl adenylate synthesized by catalytic amounts of added *E. coli* ThrRS (10 μM), no significant [^{14}C]-L-Thr incorporation was observed. This result, which contrasts with the aforementioned [^{14}C]-L-Met incorporation, might be interpreted as follows: (i) the hydroxyl group of threonine can react with the carbonyl of threonyl adenylate to form an ester bond, and as a consequence, the concentration of threonyl adenylate would become limiting in the modification of ValRS; and (ii) threonyl adenylate might remain tighly bound to the active site of ThrRS so that only slight diffusion of the donor out of the active site would occur.

As in the case of MetRS (*23*), the rate of L-valine or L-threonine incorporation into ValRS as a function of amino acid concentration varied in a hyperbolic manner, with half-saturation by L-Val and L-Thr at 62 ± 2 and 300 ± 15 μM, respectively. This behavior is somewhat unexpected because of the presence in the assay of pyrophosphatase which displaces the equilibrium of the synthetase with substrates toward 100% adenylate, as mentioned above. One explanation of this behavior has already been discussed (*23*). After adenylate synthesis, further binding of a valine or threonine molecule to their site would compete with adenylate and help to displace it from the enzyme or prevent its reassociation once it has been dissociated. Consequently, the ratio of free to bound aminoacyl adenylate would increase as a function of the saturation of the enzyme by the amino acid. Indeed, a half-saturation value of 62 ± 2 μM for L-Val in the autovalylation reaction of ValRS strongly agrees with the K_M of this amino acid in the valine-dependent [^{32}P]PP_i−ATP exchange reaction (K_M^{Val} = 70 μM in Table 3). It should be noted that K_M values of 140 μM for Val and 17 mM for Thr in the amino acid-dependent [^{32}P]PP_i−ATP exchange reaction were previously reported by Owens and Bell (*24*). Altogether, these data suggest that, to be involved in isopeptide bond formation with an acceptor protein, the adenylate must be submitted to at least one cycle of dissociation and reassociation.

The rate of Val or Thr incorporation as well as the maximum amount of valylation or threonylation of ValRS increased with the amount of enzyme in the incubation mixture, in the range of 0.5−10 μM (results not shown). In this range, the stoichiometries of labeling progressively tended to plateau values within 3−4 h corresponding to 2.1 ± 0.2 mol of [^{14}C]-L-Val and 0.063 ± 0.003 mol of [^{14}C]-L-Thr incorporated per mole of ValRS. As previously discussed (*23*), this behavior is compatible with a reaction mechanism involving either the dissociation of the aminoacyl adenylate molecules formed by the enzyme, followed by a pre-equilibrium of the reagent with the acceptor protein in the mechanism of covalent incorporation, or bimolecular contacts between a donor ValRS molecule and an acceptor one, followed by an intermolecular covalent transfer of the amino acid.

Intermolecular modification by valyl adenylate is also indicated by the observation that, upon addition to the incubation mixture, bovine serum albumin (BSA) was

covalently attached to [^{14}C]-L-Val (results not shown). Modification of ValRS with methionyl adenylate synthesized by a donor MetRS must clearly be intermolecular, as previously reported for the autoaspartylation of aspartyl-tRNA synthetase (*25*).

Finally, one cannot exclude the possibility that a part of the aminoacyl adenylate molecules formed by ValRS is involved in an in situ intramolecular covalent reaction with the enzyme at the active center.

Valylated or Threonylated Amino Acid Residues of ValRS. The tryptic digests of valylated, threonylated, or methionylated ValRS were chromatographed on a C18 column. As a control, an intact ValRS sample was submitted to trypsin digestion and RPLC separation under the same conditions as the labeled synthetase, with monitoring at 215 nm. Radioactive and/or 215 nm absorbing fractions obtained in a labeling experiment were each analyzed by MALDI mass spectrometry. The 215 nm absorbing fractions of the RPLC profile of the control were also analyzed. Distinctions between labeled and unlabeled peptides, as well as assignment of the Val-, Thr-, or Met-labeled peptides, were elucidated by comparing the molecular weights of fractions containing radioactive and/or 215 nm absorbing material to those of the corresponding fractions in the control. The molecular weight of a given aminoacylated peptide corresponded to the weight of this peptide increased by that of one [^{14}C]aminoacyl residue (weight of 134 for [^{14}C]methyl-L-Met and weight of 110 for each [^{14}C]U-L-Thr and [^{14}C]U-L-Val).

Valylated residues of ValRS were lysines 154, 162, 170, 533, 554, 593, 894, 930, and 940 (Table 1) (*26*). Threonylated residues of ValRS were lysines 162, 170, 178, 277, 291, 554, 580, 593, 861, 894, and 930. In the case of covalent methionylation, the labeled residues were lysines 118, 162, 170, 178, 277, and 938. Finally, analysis of the control ValRS sample enabled confirmation of ~90% of the amino acid sequence of the synthetase.

Only lysyl residues were found to be covalently labeled by valine, threonine, or methionine. However, other nucleophilic side chains such as the sulfhydryl group of cysteine, or the hydroxyl group of either tyrosine, serine, or threonine, are, in principle, capable of reacting with aminoacyl adenylate. The latter modifications were not evidenced in the peptides that were analyzed by MALDI-MS, but one cannot exclude the possibility that the corresponding ester linkages have been hydrolyzed during isolation of the peptides. Such a case would contrast with the aspartate system where the different types of nucleophiles were found to be aspartylated (*25*).

Alignment of ValRS Amino Acid Sequences around the Labeled Amino Acid Residues. Alignment of the available ValRS sequences (Figure 2) revealed that, among all labeled residues, only Lys-277 and Lys-554 are strictly conserved, the exception being the replacement of Lys-277 with a methionine or a tyrosine in a subgroup corresponding to the archaebacteria. In the case of the ValRS of *Rickettsia prowazekii*, the fact that this protein is very similar with archaebacterial ValRS may account for the presence of a methionine at the position of Lys-277 (*27*, *28*).

In contrast, lysines 118, 170, 291, 580, and 593 are poorly conserved at first sight (Figure 2). However, Hashimoto et al. (*29*) recently noted a 37-residue insertion downstream from the KMSKS motif which differentiates the γ-proteobacterial and eukaryotic ValRS from all others. Lysines 580 and 593 of the *E. coli* enzyme belong to this insertion. In Figure 2, we extend this comparison to new ValRS sequences. Interestingly, the 37-residue insertion is also present in the β-proteobacteria subgroup. In all cases, lysines 118, 170, 291, 580, and 593 are conserved in the insertion.

Table 1: MALDI-MS Analysis of Valylated, Threonylated, or Methionylated Peptides of ValRS[a]

labeled peptide sequence	measured weight	calcd weight	labeled residue
[^{14}C]-l-valine-labeled peptides			
^{143}FTMDEGLSNAVKEVFVR^{159}	1942.0	1942.2	Lys-154
^{160}LYKEDLIYR^{168}	1210.6	1212.4	Lys-162
^{169}GKR^{171}	357.2	359.4	Lys-170
^{529}DENGKPQVPFHTVYMTGLIRD-DEGQK^{554}	2976.8	2975.2	Lys-533
^{549}DDEGQKMSK^{557}	1037.8	1037.1	Lys-554
^{582}TGNMMQPQLADKIR^{595}	1602.5	1602.9	Lys-593
^{892}LAKEVAK^{898}	756.0	757.9	Lys-894
^{929}EKLEGYAEAK^{938}	1137.1	1137.2	Lys-930
^{939}AKLIEQQAVIAAL^{951}	1369.0	1367.6	Lys-940
[^{14}C]-l-threonine-labeled peptides			
^{160}LYKEDLIYR^{168}	1210.6	1212.4	Lys-162
^{169}GKR^{171}	358.2	359.4	Lys-170
^{172}LVNWDPKLR^{180}	1141.0	1140.3	Lys-178
^{272}GTGCVKITPAHDFNDYEVGK^{291}	2151.6	2151.4	Lys-277
^{278}ITPAHDFNDYEVGKR^{292}	1762.1	1761.9	Lys-291
^{549}DDEGQKMSK^{557}	1038.8	1037.1	Lys-554
^{560}GNVIDPLDMVDGISLPELLEKR^{581}	2424.3	2423.8	Lys-580
^{582}TGNMMQPQLADKIR^{595}	1603.0	1602.9	Lys-593
^{850}LESITVLPADDKGPVSVTK^{868}	1968.1	1969.2	Lys-861
^{892}LAKEVAK^{898}	760.3	757.9	Lys-894
^{929}EKLEGYAEAK^{938}	1136.4	1137.2	Lys-930
[^{14}C]-l-methionine-labeled peptides			
^{114}IWEWKAESGGTITR^{127}	1636.0	1633.8	Lys-118
^{160}LYKEDLIYR^{168}	1213.1	1212.4	Lys-162
^{169}GKR^{171}	358.9	359.4	Lys-170
^{172}LVNWDPKLR^{180}	1142.7	1140.3	Lys-178
^{272}GTGCVKITPAHDFNDYEVGK^{291}	2152.9	2151.4	Lys-277
^{931}LEGYAEAKAK^{940}	1081.6	1079.2	Lys-938

[a] Calculated and observed weights of the corresponding unlabeled peptides are given for molecular ions in the positive ($[M + H]^+$) ion mode. Labeled peptides showed each a weight increase, corresponding to the covalent addition of one [^{14}C]aminoacyl residue (molecular weight of 134 for [^{14}C]methyl-L-Met and 110 for each [^{14}C]U-L-Thr and [^{14}C]U-L-Val). Lysyl residues in bold and larger in size are those around which amino acid sequences of ValRS from different origins were aligned in Figure 2.

Catalytic Site where Valyl Adenylate or Threonyl Adenylate Formation Would Take Place on E. coli ValRS. Altogether, L-Val, L-Thr, and L-Met label, on one hand, lysines 861, 894, 930, 938, and 940 in the C-terminal region of ValRS (*7*). This region is situated apart from the catalytic domain located in the N-terminal half of the enzyme. Therefore, autoaminoacylation of these residues is likely to occur through diffusion of valyl adenylate or of threonyl adenylate from the ValRS active center. The observation that the maximum stoichiometry of labeling of ValRS by valine exceeds the active stoichiometry of this synthetase argues in favor of this view.

On the other hand, L-Val and L-Thr both label lysines 162 and 170. These residues are located in the N-terminal half of the enzyme. However, their location far from the catalytic center again suggests a labeling through a diffusion of the adenylate. Indeed, noncognate methionyl adenylate synthesized by a donor MetRS also labeled these two residues along with the neighboring Lys-178.

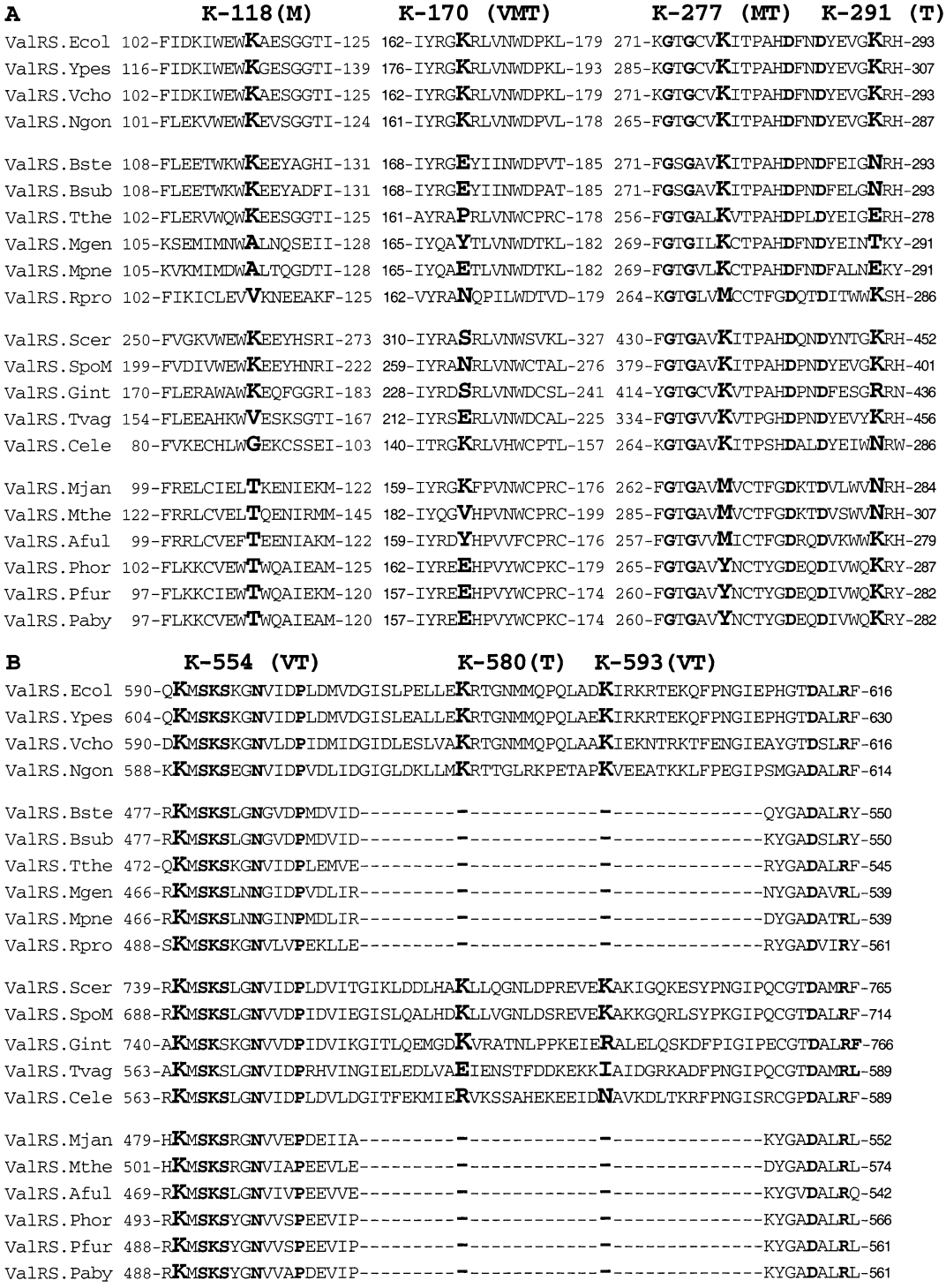

FIGURE 2: Alignment of amino acid sequences of ValRS from different origins, around the identified labeled lysyl residues of *E. coli* ValRS. Lysyl residues covalently attached to valine, threonine, or methionine as well as the corresponding amino acid residues are in bold and larger. Other conserved residues are in bold and the same size. (M), (VMT), (T), and (VT) denote the type of labeling by methionyl (M), valyl (V), or threonyl (T) adenylate. (A) Lysyl residues labeled in the N-terminal half, including the CP region of the synthetase. (B) Lysyl residues labeled in the catalytic domain containing the KMSKS consensus motif. Abbreviations of the cited organisms are as follows. For prokaryotes (first part): Ecol, *E. coli*, CG; Ypes, *Yersinia pestis*, CG; Vcho, *Vibrio cholerae*, CG; Ngon, *Neisseria gonorrheae* (all previous sequences belong to β- and γ-proteobacterial groups); Bste, *B. stearothermophilus*; Bsub, *Bacillus subtilis*, CG; Tthe, *T. thermophilus*; Mgen, *Mycoplasma genitalium*, CG; Mpne, *Mycoplasma pneumoniae*, CG; and Rpro, *R. prowazekii*, CG). For eukaryotes (second part): Scer, *Saccharomyces cerevisiae*, CG; SpoM, *Schizosaccharomyces pombe* mitochondria; Gint, *Giardia intestinalis*; Tvag, *Trichomonas vaginalis*; and Cele, *Caenorhabditis elegans*. For archaebacteria (third part): Mjan, *Methanococcus jannaschii*, CG; Mthe, *Methanobacterium thermoautotrophicum*, CG; Aful, *Archaeoglobus fulgidus*, CG; Phor, *Pyrococcus horikoshii*, CG; Pfur, *Pyrococcus furiosus*; and Paby, *Pyrococcus abyssi*. (CG) is for complete genomes (sequences in GenBank). All other eubacterial sequences are from several genomic programs in progress and can be accessed via a general Web page (http://www.infobiogen.fr/doc/data/uncomplete_genome.html).

L-Val and L-Thr both label lysines 554 and 593. These two lysines do not react with methionyl adenylate, in good agreement with the incapacity of ValRS to activate L-Met. By comparison with the three-dimensional structure of *T.*

thermophilus ValRS, these two lysines are located in the catalytic cleft. Lys-554 is the first lysine of the KMSKS motif of class I aminoacyl-tRNA synthetases (*5*, *30*, *31*). In *E. coli* MetRS, the second lysyl residue of this motif is Lys-335 which was covalently labeled by the periodate-oxidized CCA arm of initiator $tRNA^{Met}$ (*30*). Later on, Lys-335 of *E. coli* MetRS was shown by site-directed mutagenesis to be involved in the stabilization of the transition state during the synthesis of methionyl adenylate (*32*). In other affinity labeling studies, ATP analogues have been used as active site-directed reagents of lysyl residues at the nucleotide binding site of MetRS and ValRS from *E. coli* (*33*, *34*). Pyridoxal 5′-triphospho-5′-adenosine (ATP-PL) and pyridoxal 5′-phosphate (PLP) affinity label lysines 332, 335 (KMSKS), and 402 of MetRS, as well as lysines 554, 557, 559 (KMSKSK), and 593 of ValRS (*33*, *34*). Comparison of the primary structure of MetRS around Lys-402 to that of ValRS around Lys-593 had revealed significant similarity, with four identical residues and two conservative replacements out of 11 amino acid residues. It was proposed that the common consensus KMSKS motif as well as Lys-402 of MetRS and the corresponding Lys-593 of ValRS represent the subsite of the pyrophosphate moiety of ATP at the catalytic center of these two synthetases (*33*). The observation that lysines 554 and 593 of ValRS are autoaminoacylated by either L-Val or L-Thr supports this conclusion.

Putative Editing Site where L-Threonine Might Bind on E. coli ValRS. Apart from Lys-178, the only lysyl residue which was labeled by both L-Thr and L-Met is Lys-277. This residue belongs to the so-called connecting peptide (CP1) which is involved in the editing in some class I aminoacyl-tRNA synthetases (*6*). Consequently, we hypothesize that labeling of Lys-277 by noncognate L-Thr, which is involved via editing, or by methionine might result from nucleophilic attack of the carbonyl group of threonyl adenylate or of methionyl adenylate at the editing site of the synthetase.

In the report by Fukai et al. (*7*), the crystal structure of ValRS from *T. thermophilus* shows two binding sites for L-Thr, one on the aminoacylation domain and another one on the editing domain. Interestingly, in the editing site, the side chain of Lys-270 (corresponding to Lys-277 of *E. coli* ValRS) is close to that of bound L-Thr. A functional role for Lys-277 in editing was also suggested by the comparison of the primary structure of *E. coli* ValRS around Lys-277 and that of *T. thermophilus* ValRS around Lys-270 with those of all the ValRS and IleRS sequenced so far. A matching of Lys-277 of *E. coli* ValRS with His-319 of *T. thermophilus* IleRS was noticed. The latter histidine is supposed to be involved in the editing by IleRS (*9*). Finally, Lys-277 was one mutational target in the selection procedure designed to isolate ValRS that charge $tRNA^{Val}$ with cysteine (*8*).

Effect of Aminoacylation on ValRS Activity. E. coli ValRS having incorporated 2.1 mol of [^{14}C]Val per mole of enzyme was inactivated by 95% in the [^{32}P]PP_i−ATP exchange or $tRNA^{Val}$ aminoacylation activities. The fact that such a 2.1:1 [^{14}C]-L-Val:ValRS stoichiometry is higher than the active stoichiometry of this synthetase (1 mol of valine/mol enzyme) can be explained by a distribution of the amino acid between lysine side chains both in the active site and in the C-terminal region of the enzyme.

In the cases of ValRS having incorporated 0.063 mol of [^{14}C]-L-Thr or 0.65 mol of [^{14}C]-L-Met per mole of enzyme, the two enzyme activities remained unchanged (results not shown). In the report by Nureki et al. (*9*, *35*), where the editing site of *T. thermophilus* IleRS was identified by determining the structure of a cocrystal with valine, this amino acid was bound to two sites, the active site and the editing site. Covalent attachment of threonine in both the catalytic site and the putative editing site of ValRS may therefore be expected. However, since a maximum of only 0.063 mol of [^{14}C]-L-Thr could be incorporated by 1 mol of ValRS, the critical Lys-554 and Lys-593 in the catalytic site of this synthetase may be assumed to remain marginally labeled. Consequently, the enzyme activities stay unchanged upon threonylation. On the other hand, upon incorporation of [^{14}C]-L-Met, only lysyl residues in the putative editing site would be labeled because the diffusing methionyl adenylate cannot be accommodated inside the active site. Accordingly, the enzyme activities must be insensitive to modification by methionine.

Table 2: Kinetic Parameters of Wild-Type and His-Tagged *E. coli* ValRS.

enzyme	k_{cat} (s^{-1}) for [^{32}P]PP_i−ATP exchange[a]	$tRNA^{Val}$ aminoacylation with valine[b]		hydrolysis of Thr-$tRNA^{Val}$[c] (s^{-1})
		k_{cat} (s^{-1})	$K_M{}^{tRNA}$ (μM)	
wild type	51	1.7	0.08	370×10^{-3}
His-tagged	50	1.5	0.1	180×10^{-3}

[a] Initial rates of [^{32}P]PP_i−ATP exchange were measured at 25 °C in the presence of 20 mM Tris-HCl (pH 7.5), 2 mM valine, 2 mM ATP, 2 mM [^{32}P]PP_i, 7 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 50 μg/mL BSA. [b] Initial rates of $tRNA^{Val}$ aminoacylation were measured at 25 °C in the presence of 20 mM Tris-HCl (pH 7.5), 7.7 μM [^{14}C]valine, 2 mM ATP, 7 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 50 μg/mL BSA, and various concentrations of $tRNA^{Val1}$. [c] Initial rates of Thr-$tRNA^{Val}$ deacylation were measured at 25 °C in the presence of 20 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 50 μg/mL BSA, 0.25 μM [^{14}C]Thr-$tRNA^{Val1}$, and 10 nM purified wild-type ValRS or 20 nM mutant ValRS.

Characterization of the K277A ValRS Mutant. To probe the role of the Lys-277 side chain in editing, we generated an alanine mutant at position 277 (K277A) by site-directed mutagenesis. First attempts to subclone the K277A mutant gene into phagemid pBluescript and to overexpress the corresponding mutant ValRS in *E. coli* strain JM101Tr were unsuccessful, suggesting that a high level of production of the K277A mutant ValRS was lethal.

To overcome the problem of cell growth, the mutant *val*S gene was introduced into plasmid pTrc99A, under control of the inducible *trc* promoter. In addition, this gene was fused with a DNA sequence encoding an N-terminal six-histidine tag. Thus, during the affinity chromatographic step, the mutant enzyme was retained on the Ni−NTA agarose column thanks to the tag, while the wild-type chromosomally encoded *E. coli* enzyme was not. As a consequence, combination of this affinity chromatographic step with ion exchange on a Q-HiLoad column allowed us to obtain the mutant enzyme free from contaminants.

To determine whether the His tag of the K277A mutant interfered with catalytic and editing activities, a wild-type His-tagged ValRS was also produced. The kinetic properties of this enzyme were compared to those of wild-type ValRS. As shown in Table 2, the kinetic parameters of both forms

Table 3: Comparison of Kinetic Data for His-Tagged Wild-Type and K277A Mutant ValRS

	[^{32}P]PP$_i$−ATP exchange[a]			tRNA^{Val} aminoacylation with valine [b]			rate of aa-tRNA hydrolysis (s^{-1})[c]	
His-tagged enzyme	k_{cat} (s^{-1})	K_M^{Val} (μM)	K_M^{ATP} (mM)	k_{cat} (s^{-1})	K_M^{tRNA} (μM)	K_M^{Val} (μM)	Thr-tRNA^{Val}	Val-tRNA^{Val}
wild type	50	70	0.11	1.5	0.10	47	180×10^{-3}	0.16×10^{-3}
K277A	57	72	0.11	0.064	0.26	1.9	8×10^{-3}	6.8×10^{-3}

[a] Initial rates of [^{32}P]PP$_i$−ATP exchange were measured at 25 °C in the presence of 20 mM Tris-HCl (pH 7.5), 2 mM [^{32}P]PP$_i$, 7 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 50 μg/mL BSA. The K_M value for ATP was measured in the presence of 2 mM valine and various concentrations of ATP. The K_M value for valine was measured in the presence of 2 mM ATP and various concentrations of valine. [b] Initial rates of tRNA^{Val} aminoacylation were measured at 25 °C in the presence of 20 mM Tris-HCl (pH 7.5), 2 mM ATP, 7 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 50 μg/mL BSA. The K_M value for valine was measured in the presence of 8 μM tRNA^{Val1} and various concentrations of [^{14}C]valine. The K_M value for tRNA^{Val} was measured in the presence of 7.7 μM [^{14}C]valine and various concentrations of tRNA^{Val1}. [c] Initial rates of Thr-tRNA^{Val} deacylation were measured at 25 °C in the presence of 20 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 50 μg/mL BSA, 0.25 μM [^{14}C]Thr-tRNA^{Val1}, and either 10 nM purified wild-type ValRS or 20 nM mutant ValRS. Enzymatic deacylation of Val-tRNA^{Val} was assayed under the same conditions, except that 0.77 μM [^{14}C]Val-tRNA^{Val1} and 100 nM purified wild-type or mutant ValRS was used.

of ValRS were similar, suggesting that the tag did not significantly interfere with activity.

As shown in Table 3, mutation of Lys-277 did not affect the parameters of the valine-dependent [^{32}P]PP$_i$−ATP exchange reaction. In the tRNA aminoacylation reaction, we observed a marked sensitivity of the activity of the K277A mutant enzyme to free magnesium (results not shown). When the magnesium level was varied at a fixed level of ATP , optimal activity was reached for a $MgCl_2$ concentration nearly equal to that of ATP. However, beyond this stoichiometry, addition of excess $MgCl_2$ drastically decreased the activity. For instance, with 2 mM ATP and 7 mM $MgCl_2$, the activity of the mutant protein was 7-fold lower than in the presence of 2 mM ATP and 2 mM $MgCl_2$. With native ValRS, activity was insensitive to excess $MgCl_2$, at least up to 30 mM.

The abnormal sensitivity to metal ion of the K277A species precluded reliable measurement of the K_M for ATP in the tRNA aminoacylation reaction. In contrast, kinetic parameters for valine and tRNA could be obtained in the presence of a $MgCl_2$ concentration exceeding that of ATP by 5 mM (Table 3). Under such conditions, the k_{cat} (0.064 s^{-1}) and K_M^{Val} (1.9 μM) values of the K277A enzyme were quite far from the corresponding parameters measured with wild-type ValRS (1.5 s^{-1} and 47 μM, respectively). However, the sensitivity of the mutant to 5 mM free magnesium can account for a relatively low activity. Indeed, if 2 mM ATP and 2 mM $MgCl_2$ were added, without magnesium in excess, the k_{cat} associated with the K277A species reached 1.7 s^{-1}. In parallel, the K_M value for valine increased to 11 μM, a value much closer to that of the K_M value for the wild-type enzyme (47 μM). Therefore, at this stage, we may conclude that under carefully controlled ionic conditions, the K277A mutation does not interfere with the catalytic center of the synthetase.

To probe the role of K277A in posttransfer editing, we followed the deacylation of [^{14}C]Thr-tRNA^{Val} by the mutant enzyme. As shown in Figure 3 and Table 3, the posttransfer editing rate of K277A ValRS was significantly lower (by roughly 20−25-fold) than that observed for the wild-type enzyme. This result might reflect the inability of the K277A mutant ValRS to release noncognate amino acids that have been incorrectly charged onto tRNA^{Val}.

In some cases, aminoacyl-tRNA synthetases that are mutated in their editing site are found to hydrolyze tRNAs correctly charged with the cognate amino acid (*11*, *13*, *36*). To know whether the K277A mutant was capable of editing

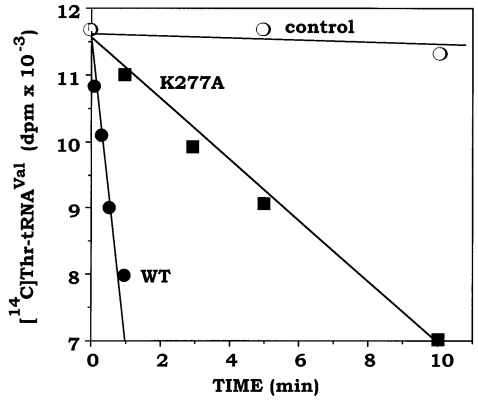

FIGURE 3: Deacylation of Thr-tRNA^{Val} by wild-type and K277A ValRS. Deacylation of Thr-tRNA^{Val} was assayed at 25 °C in the presence of 20 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 50 μg/mL BSA, 0.25 μM [^{14}C]-Thr-tRNA^{Val1}, and 10 nM purified wild-type ValRS (●) or 20 nM mutant ValRS (■). Control experiments (○) were carried out without enzyme.

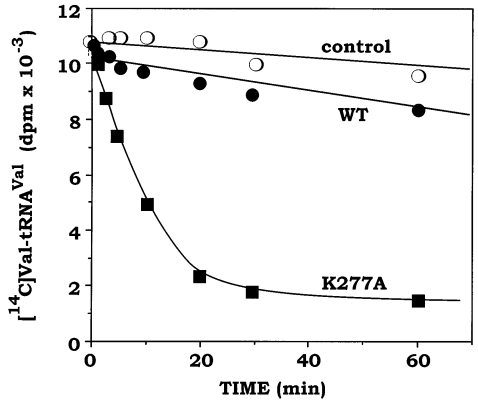

FIGURE 4: Deacylation of Val-tRNA^{Val} by wild-type and K277A ValRS. Deacylation of Val-tRNA^{Val} was assayed at 25 °C in the presence of 20 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 50 μg/mL BSA, 0.77 μM [^{14}C]-Val-tRNA^{Val1}, and 100 nM purified wild-type ValRS (●) or mutant ValRS (■). Control experiments (○) were carried out without enzyme.

tRNA^{Val} esterified with cognate valine, [^{14}C]Val-tRNA^{Val} was incubated with either wild-type ValRS or the K277A mutant. In the presence of wild-type ValRS, [^{14}C]Val-tRNA^{Val} was hydrolyzed at a rate only slightly faster than that measured in the control reaction without enzyme (Figure 4 and Table 3). In contrast, a 43-fold more rapid deacylation of Val-tRNA^{Val} was induced by the K277A species (Figure 4 and

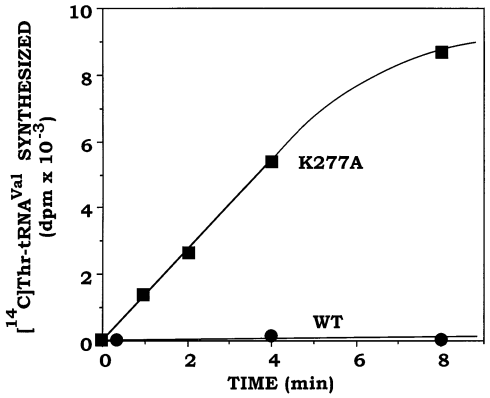

FIGURE 5: Misacylation of $tRNA^{Val}$ with threonine. Wild-type (●) or K277A mutant ValRS (■) was incubated at 160 nM in the presence of 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 50 μg/mL BSA, 8 μM $tRNA^{Val}$, 2 mM ATP, and 4.7 μM [^{14}C]-L-threonine (241.6 mCi/mmol). After the mixtures had remained for various times at 25 °C, reactions (100 μL volume) were quenched by addition of 5% TCA and the mixtures filtered and quantified in a scintillation counter.

Table 3). These results show that the K277A substitution has altered amino acid discrimination in the editing active site, resulting in hydrolysis of the correctly charged cognate product. At this stage, one can imagine that the strictly conserved Lys-277 residue acts as a critical determinant of the ValRS editing active site that (i) blocks charged valine from being bound and subsequently hydrolyzed but (ii) contributes to the removal of charged threonine. The observation that Thr-$tRNA^{Val}$ (0.25 μM) and Val-$tRNA^{Val}$ (0.77 μM) substrates were deacylated at similar rates by catalytic amounts of K277A ValRS supports this idea (Figures 3 and 4 and Table 3). Similarly, the approximately 1100-fold difference observed between the rates of deacylation of Thr-$tRNA^{Val}$ and Val-$tRNA^{Val}$ by wild-type ValRS (Table 3) is consistent with a functional role for Lys-277 in amino acid discrimination. Altogether, these results might explain why a high level of constitutive overexpression of the K277A mutant ValRS in *E. coli* could not be obtained.

Wild-type ValRS is known to misactivate L-Thr and to generate Thr-$tRNA^{Val}$ which normally is hydrolyzed by the ValRS editing activity (*3*). Therefore, wild-type ValRS is incapable of generating Thr-$tRNA^{Val}$ in significant amounts. As shown in Figure 5, the K277A mutation enabled facile mischarging of $tRNA^{Val}$ with threonine. These results suggest that Lys-277 might represent one nucleophilic amino acid residue involved in the hydrolysis of misacylated tRNA.

Editing Site of the ValRS, IleRS, and LeuRS Families. On the basis of crystallographic studies and mutational analyses of the CP1 regions of ValRS (*7*, *8*), IleRS (*9*, *10*), and LeuRS (*12*, *13*), the editing site of these synthetases is currently the object of numerous investigations.

The crystal structure of IleRS from *T. thermophilus* shows two amino acid sites, one for aminoacylation with Ile and one for editing of misactivated Val (*9*, *35*). In the editing site, six residues (Thr-228, Thr-229, Thr-230, Thr-233, Asn-237, and His-319) are located close together, in a way that is reminiscent of the catalytic triads of hydrolases. Among these, only Thr-228, Thr-230, Thr-233, and His-319 are strictly conserved in IleRS enzymes from different bacterial origins (Figure 6). Of the four latter, only Thr-228 is strictly

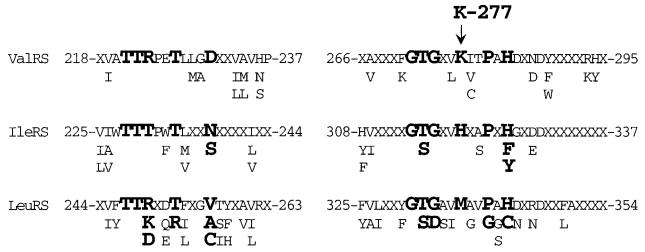

FIGURE 6: Conserved amino acid residues belonging to the eubacterial ValRS, IleRS, and LeuRS families in the two canonical CP regions related to the editing site. Amino acid residues strongly conserved and/or discussed in the text are in bold. The numbering is relative to the sequences of *E. coli* ValRS, *T. thermophilus* IleRS, and *E. coli* LeuRS.

conserved in the primary structure of all IleRS, LeuRS, and ValRS enzymes sequenced so far, among all kingdoms, suggesting that this residue might play a crucial role in the editing by the synthetases specific for large aliphatic amino acids. In contrast, Asn-237 and His-319 are not conserved in the ValRS and LeuRS sequences (Figure 6). In the ValRS family, they are replaced with an aspartic acid (Asp-230 in *E. coli* ValRS) and a lysine (Lys-277 in *E. coli* ValRS), respectively (Figure 6). In LeuRS, Asn-237 is replaced with either Val, Ala, or Cys, while His-319 is replaced with Met (Figure 6). Therefore, the involvement of a mechanism analogous to the charge relay system in the editing site of the ValRS and LeuRS families is less probable.

Interestingly, crystal structures of *T. thermophilus* LeuRS (*12*), *T. thermophilus* ValRS (*7*), *T. thermophilus* IleRS (*9*), and *Streptomyces aureus* IleRS (*37*) exhibit structural homology within their CP1 domain, which suggests an overlap in hydrolytic editing mechanisms. Whatever the mechanism, a nucleophile is needed in the editing site to achieve hydrolysis of an undesired product at the level of the carbonyl group thereof. This nucleophile is requested to be unprotonated to attack the carbonyl group. In the case of the ValRS family, our results are compatible with Lys-277 being this nucleophile.

Recently, we used bromomethyl ketone derivatives of valine (VBMK), isoleucine (IBMK), phenylalanine (FBMK), and norleucine (NleBMK) to identify the binding sites for L-valine or for noncognate amino acids on *E. coli* ValRS. On one hand, Cys-424 was specifically labeled by VBMK, suggesting that this residue represents the locus where L-valine activation takes place. On the other hand, Cys-275 and His-282 were labeled by IBMK, FBMK, and NleBMK, but not by VBMK, suggesting that these residues might be located at or near the binding site for noncognate amino acids (*28*). In addition, VBMK-labeled ValRS deacylated [^{14}C]-Thr-$tRNA^{Val}$ as efficiently as the control intact ValRS, while IBMK-labeled ValRS was no more capable of hydrolyzing this misacylated $tRNA^{Val}$, suggesting that alkylation of Cys-275 and/or His-282 impaired the posttransfer editing activity of an IBMK-labeled synthetase (*28*). It was proposed that these residues might be involved in the editing activity of ValRS. Since proximity among several nucleophilic amino acid residues can help to deprotonate either of these residues in some way, Cys-275 and His-282, located only two and five residues from Lys-277, respectively, might form with the latter a cluster of nucleophiles participating in the editing reaction.

CONCLUSION

L-Val and L-Thr, activated by ValRS itself, both label Lys-554 (of the ^{554}KMSKS^{558} consensus) and Lys-593 in the catalytic site of the enzyme.

On the other hand, Lys-277 of the CP1 region is labeled by L-Thr and by L-Met, but not by L-Val, suggesting that this residue is located at or near the editing site of the synthetase. Attachment of threonine to lysyl residues in both the catalytic and the putative editing site reflects the existence of two binding sites for this isosteric noncognate substrate. As proposed first by Fersht and Kaethner (*3*), and more recently by Nureki et al. (*9*, *35*), mischarged L-Thr would move from the catalytic site to the editing site via a conformational change that is likely to take place during the editing reaction.

Alignment of the available ValRS sequences revealed that Lys-277 was strictly conserved (with the exception concerning its replacement with a Met or a Tyr in the archaebacteria), suggesting that this residue might be functionally significant.

In a recent report by Fukai et al. (*7*), the crystal structure of ValRS from *T. thermophilus* showed two sites for L-Thr, one on the aminoacylation domain and one on the editing domain. Interestingly, in the editing site, the side chain of Lys-270 (corresponding to Lys-277 of *E. coli* ValRS) is located in the proximity of that of L-Thr.

The role of Lys-277 in editing was evaluated by site-directed mutagenesis. K277A ValRS exhibited a significantly lower posttransfer Thr-tRNA^{Val} editing rate than the wild-type enzyme. In addition, the K277A substitution alters amino acid discrimination in the editing active site, resulting in hydrolysis of the correctly charged cognate product. Finally, the K277A mutant enzyme significantly misacylated tRNA^{Val} with Thr to give Thr-tRNA^{Val}, whereas the wild-type enzyme did not produce detectable mischarged tRNA^{Val}.

Altogether, our results designate Lys-277 as a likely residue involved, through nucleophilic attack of a carbonyl group, in the hydrolysis of misacylated tRNA, in the editing site of ValRS.

ACKNOWLEDGMENT

All data analyses have been performed with the help of Infobiogen bioinformatic resources (http://www.infobiogen.fr).

REFERENCES

1. Loftfield, R. B., and Vanderjagt, D. (1972) *Biochem. J. 128*, 1353−1356.
2. Schmidt, E., and Schimmel, P. (1994) *Science 264*, 265−267.
3. Fersht, A. R., and Kaethner, M. M. (1976) *Biochemistry 15*, 3342−3346.
4. Fersht, A. R. (1977) *Biochemistry 16*, 1025−1030.
5. Hountondji, C., Lederer, F., Dessen, P., and Blanquet, S. (1986) *Biochemistry 25*, 16−21.
6. Starzyk, R. M., Webster, T. A., and Schimmel, P. (1987) *Science 237*, 1614−1618.
7. Fukai, S., Nureki, O., Sekine, S., Shimada, A., Tao, J., Vassylyev, D. G., and Yokoyama, S. (2000) *Cell 103*, 793−803.
8. Döring, V., Mootz, H. D., Nangle, L. A., Hendrickson, T. L., de Crécy-Lagard, V., Schimmel, P., and Marlière, P. (2001) *Science 292*, 501−504.
9. Nureki, O., Vassylyev, D. G., Tateno, M., Shimada, A., Nakama, T., Fukai, S., Konno, M., Hendrickson, T. L., Schimmel, P., and Yokoyama, S. (1998) *Science 280*, 578−582.
10. Hendrickson, T. L., Nomanbhoy, T. K., and Schimmel, P. (2000) *Biochemistry 39*, 8180−8186.
11. Hendrickson, T. L., Nomanbhoy, T. K., de Crécy-Lagard, V., Fukai, S., Nureki, O., Yokoyama, S., and Schimmel, P. (2002) *Mol. Cell 9*, 353−362.
12. Cusack, S., Yaremchuk, A., and Tukalo, M. (2000) *EMBO J. 19*, 2351−2361.
13. Mursinna, R. S., Lincecum, T. L., Jr., and Martinis, S. A. (2001) *Biochemistry 40*, 5376−5381.
14. Mechulam, Y., Schmitt, E., Panvert, M., Schmitter, J. M., Lapadat-Tapolsky, M., Meinnel, T., Dessen, P., Blanquet, S., and Fayat, G. (1991) *Nucleic Acids Res. 19*, 3673−3681.
15. Blanquet, S., Fayat, G., and Waller, J. P. (1974) *Eur. J. Biochem. 44*, 343−351.
16. Lawrence, F., Blanquet, S., Poiret, M., Robert-Gero, M., and Waller, J. P. (1973) *Eur. J. Biochem. 36*, 234−243.
17. Brevet, A., Chen, J., Lévêque, F., Plateau, P., and Blanquet, S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8275−8279.
18. Sayers, J. R., Schmidt, W., and Eckstein, F. (1988) *Nucleic Acids Res. 16*, 791−802.
19. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
20. Fromant, M., Plateau, P., and Blanquet, S. (2000) *Biochemistry 39*, 4062−4067.
21. Brevet, A., Chen, J., Lévêque, F., Blanquet, S., and Plateau, P. (1995) *J. Biol. Chem. 270*, 14439−14444.
22. Meinnel, T., Mechulam, Y., and Fayat, G. (1988) *Nucleic Acids Res. 16*, 8095−8096.
23. Gillet, S., Hountondji, C., Schmitter, J. M., and Blanquet, S. (1997) *Protein Sci. 6*, 2426−2435.
24. Owens, S. L., and Bell, F. E. (1970) *J. Biol. Chem. 245*, 5515−5523.
25. Mejdoub, H., Kern, D., Giegé, R., Ebel, J. P., Boulanger, Y., and Reinbolt, J. (1987) *Biochemistry 26*, 2054−2059.
26. Borgford, T. J., Brand, N. J., Gray, T. E., and Fersht, A. R. (1987) *Biochemistry 26*, 2480−2486.
27. Andersson, S. G., Zomorodipour, A., Andersson, J. O., Sicheritz-Pontén, T., Alsmark, U. C. M., Podowski, R. M., Näslund, A. K., Eriksson, A. S., Winkler, H. H., and Kurland, C. G. (1998) *Nature 396*, 133−140.
28. Hountondji, C., Beauvallet, C., Dessen, P., Hoang-Naudin, C., Schmitter, J. M., Pernollet, J. C., and Blanquet, S. (2000) *Eur. J. Biochem. 267*, 4789−4798.
29. Hashimoto, T., Sanchez, L. B., Shirakura, T., Müller, M., and Hasegawa, M. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 6860−6865.
30. Hountondji, C., Blanquet, S., and Lederer, F. (1985) *Biochemistry 24*, 1175−1180.
31. Hountondji, C., Dessen, P., and Blanquet, S. (1986) *Biochimie 68*, 1071−1078.
32. Mechulam, Y., Dardel, F., Le Corre, D., Blanquet, S., and Fayat, G. (1991) *J. Mol. Biol. 217*, 465−475.
33. Hountondji, C., Schmitter, J. M., Fukui, T., Tagaya, M., and Blanquet, S. (1990) *Biochemistry 29*, 11266−11273.
34. Kalogerakos, T., Hountondji, C., Berne, P. F., Dukta, S., and Blanquet, S. (1994) *Biochimie 76*, 33−44.
35. Nureki, O., Vassylyev, D. G., Tateno, M., Shimada, A., Nakama, T., Fukai, S., Konno, M., Hendrickson, T. L., Schimmel, P., and Yokoyama, S. (1999) *Science 283*, 459.
36. Schmidt, E., and Schimmel, P. (1995) *Biochemistry 34*, 11204−11210.
37. Silvian, L. F., Wang, J., and Steitz, T. A. (1999) *Science 285*, 1074−1077.

BI0205101