Plasticity of Recognition of the 3’-End of Mischarged tRNA by Class I Aminoacyl-tRNA Synthetases*

Received for publication, February 28, 2002
Published, JBC Papers in Press, March 28, 2002, DOI 10.1074/jbc.M202023200

Brian E. Nordin‡ and Paul Schimmel§
From The Skaggs Institute for Chemical Biology and the Departments of Molecular Biology and Chemistry, The Scripps Research Institute, La Jolla, California 92037

Certain aminoacyl-tRNA synthetases prevent potential errors in protein synthesis through deacylation of mischarged tRNAs. For example, the close homolog isoleucyl-tRNA synthetase (IleRS) and valyl-tRNA synthetase (ValRS) deacylate Val-tRNA\textsubscript{Ile} and Thr-tRNA\textsubscript{Val}, respectively. Here we examined the chemical requirements at the 3’-end of the tRNA for these hydrolysis reactions. Single atom substitutions at the 2’- and 3’- hydroxyls of a variety of mischarged RNAs revealed that, while acylation is at the 2’-OH for both enzymes, IleRS catalyzes deacylation specifically from the 3’-OH and not from the 2’-OH. In contrast, ValRS can deacylate non-cognate amino acids from the 2’-OH. Moreover, for IleRS the specificity for a 3’-O-methyl moiety. Cumulatively, these and other results suggest that the editing sites of these class I aminoacyl-tRNA synthetases have a degree of inherent plasticity for substrate recognition. The ability to adapt to subtle differences in mischarged RNAs may be important for the high accuracy of aminoacylation.

The genetic code is based on the accurate aminoacylation of tRNAs by aminoacyl-tRNA synthetases (1, 2). These enzymes synthesize aminoacyl-tRNA in two steps. The amino acid is first reacted with ATP to give an activated aminoacyl adenylate, and then transsterified to the 3’-end of the tRNA. Aminoacyl-tRNA synthetases must precisely recognize both amino acid and tRNA substrates to yield the correct product. While the structural diversity of tRNA molecules allows for rigorous selection based on RNA-protein interactions, differentiating between closely related amino acids is more challenging. Years ago, Pauling (3) noted the intrinsic difficulty for isoleucyl-tRNA synthetase in the recognition of isoleucine over valine through simple binding interactions.

Valine, which differs from isoleucine by a single methylene unit, is activated by Escherichia coli IleRS\textsuperscript{1} only 180-fold less efficiently than isoleucine (4). However, the substitution of valine for isoleucine at isoleucine codons in the cell is less than 1 in 3000 (5). The increased specificity is a result of the RNA-dependent editing of misactivated valine by IleRS (6, 7). A highly related class I aminoacyl-tRNA synthetase, valyl-tRNA synthetase, faces a similar dilemma in the accurate aminoacylation of tRNA\textsubscript{Val}. Threonine, an isostere of valine, is activated at a rate 250-fold reduced from that of valine (8). Like IleRS, ValRS prevents the misincorporation of threonine into proteins through the RNA-dependent editing of misactivated threonine (9).

These reactions strictly require the presence of the cognate tRNA (6, 10). In the absence of tRNA, the enzymatically generated misactivated adenylates remain in the active site, sequestered from hydrolysis. Upon addition of cognate tRNA the misactivated amino acids are hydrolyzed, regenerating the free tRNA and amino acid, while converting 1 equivalent of ATP to AMP. A prominent mechanism for editing misactivated amino acids is the rapid hydrolysis of transiently mischarged tRNA (7, 9). This reaction is catalyzed at a second active site on IleRS and ValRS. This site is located within a large insertion (termed CP1) into the canonical class I aminoacyl-tRNA synthetase active-site fold. The CP1 domain as an isolated polypeptide hydrolyzes its cognate mischarged tRNA (11). Crystallographic analysis of Thermus thermophilus IleRS pinpointed the editing site to a pocket of essentially invariant amino acids within CP1 located ~30 Å from the aminoacylation active site (12). This site binds valine but sterically excludes isoleucine. A co-crystal structure of Staphylococcus aureus IleRS with tRNA\textsubscript{Ile} suggested how the tRNA may place its 3’-end in the editing site (13). However, specific interactions in the editing site could not be observed. More recently, the co-crystal structure of T. thermophilus ValRS bound to tRNA\textsubscript{Val} demonstrated a similar mode of tRNA binding (14). Although a mischarged amino acid could be modeled at the end of the tRNA, neither this model nor mutational analysis established a hydrolytic mechanism (15, 16).

Early work demonstrated an important role for the 3’-end of tRNA\textsubscript{Ile} and tRNA\textsubscript{Val} in editing (17–19). However, the exact nature of this role was not determined. Model studies of uncharged ester hydrolysis demonstrated that a cis-hydroxyl stimulates hydrolysis, likely via intramolecular hydrogen bonding (20). While its effect may be important for the efficiency of deacylation, the importance of the terminal hydroxyls could be related to the rapid transacylation known to occur between the two cis-hydroxyls (with a rate of ~10\textsuperscript{-3} s\textsuperscript{-1}) (21). Thus, while aminoacylation is specific to a particular hydroxyl (2’-OH in the cases of IleRS and ValRS (22)) deacylation could potentially occur from either the 2’- or 3’-OH group.

To address issues that could not be taken up in earlier work because of then existing technical limitations, we constructed a variety of mischarged RNA substrates having different substitutions at the positions of the terminal hydroxyl groups. Using these substrates we were able to test directly whether deacy-
loration occurred from a specific hydroxyl, whether transacylation from the 2'- to the 3'-OH was required for deacylation, and finally, whether a clear chemical role for vicinal hydroxyls could be identified.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification—**Wild-type E. coli IleRS was overexpressed in E. coli strain MV1184 from plasmid pKS21, which contains the gene for IleRS under control of the lac promoter (23). The T222P mutant of E. coli IleRS was overexpressed in E. coli strain PS2766 (Δilv, Δi1S203:kan) from plasmid pVDC434, a derivative of pBAD18 containing the gene for T222P E. coli IleRS under the control of an arabinosin inducible promoter (24). Purification of the wild-type and mutant IleRS was as described previously (25). IleRS concentrations were determined by active site titration (26).

Wild-type E. coli ValRS was overexpressed as a C-terminal His6-tagged protein in E. coli strain BL21 (DE3) from a pET-21b (Novagen, Madison, WI) derivative containing the gene for ValRS (27). Standard protocols were used for purification. The T222P mutant of E. coli ValRS was overexpressed in E. coli strain PS2801 (ΔvalS::kan) from plasmid pVDC447, a pBAD18 derivative containing the gene for T222P ValRS (28). Purification was essentially identical to the protocol used for IleRS (24). The plasmid pVDC434 in the pBAD18 derivative and the pBAD18 derivative containing the wild-type IleRS gene, which is in the editing site and do not affect aminoclaylation activity with valine (Saccharomyces cerevisiae) ValRS were overexpressed as C-terminal His6-tagged proteins in the yeast strain CW1, which has the chromosomal copy of ValRS deleted.2 Both proteins were purified using standard methods for His6-tagged proteins. ValRS concentrations were determined by the Bradford dye-binding assay.

**RNA Substrates—**Mature E. coli tRNAVal (GAU) was isolated from E. coli strain MV1184 containing the plasmid pE3S00, which allows for the isopropyl-1-thio-D-galactopyranoside-inducible overexpression of tRNAval (30). Purification was as described previously (31). The resulting preparations of tRNAval were generally comprised of 50-70% tRNAval with the remainder being other cellular tRNAs. Transcripts of tRNAval missing the terminal A76 nucleotide (ΔA76 tRNAval) were found to be superior to full-length transcripts for 3'-end modification using tRNA NTase. The plasmid pPET-22-CCA, which encodes a C-terminal His6-tagged E. coli tRNA nucleotidyltransferase (tRNA NTase) from the Hecht laboratory was used exclusively for the preparation of tRNAs charged with cognate amino acid. Preparative mischarging of mature tRNAval and the related 3'-end variants of tRNAval was accomplished using two primary techniques. Because IleRS and ValRS both charge the 2'-OH, preparing mischarged RNA substrates that have modified 2'-ends is a challenge, especially when conditions where this mischarging could take place revealed that, under conditions of no monovalent cations and 30% dimethyl sulfoxide (Me2SO), yeast ValRS had a low, but easily detectable mischarging activity toward 2'-dAT6 tRNAVal. Additionally, it was found that the T379A,T380A double mutant yeast ValRS had an equivalent activity under these conditions. (Larger quantities of the mutant ValRS were available to be used in mischarging preparations.) Preparative mischarging reaction conditions were 25 mM Tris-HCl (pH 8.5), 10 mM MgCl2, 30% Me2SO, 1 mM ATP, 10 mM inorganic pyrophosphatase, 5 μM [3H]Valine (23 Ci/mmol), 500 mM yeast ValRS (or double mutant), and 5 μM tRNA or 100 μM minihelix. Reactions were incubated at room temperature for 4–6 h, then extracted with phenol/chloroform (1:1) twice, ethanol precipitated, and resuspended in 10 mM sodium acetate (pH 4.5). This method was used for mischarging tRNAval transcripts, minihelices, and their respective 3'-end variants. By using transcripts and synthetic RNAs it could be guaranteed there was no trace contamination of tRNAval and minihelixval RNA species.

For the preparative mischarging of mature tRNAval and the related 3'-end variants, an enzyme that has no cross-reactivity with any potential tRNAval RNA in vivo was necessary. The 2'-deoxytRNAval requirement was met by using an editing-deficient mutant (T224P) (16) of E. coli IleRS under standard conditions where tRNA recognition is intact. Mischarging reactions were performed in 20 μM HEPES (pH 7.5), 100 μM EDTA, 150 mM NaCl, 10 mM MgCl2, 1 mM ATP, 10 mM inorganic pyrophosphatase, and 5–10 μM [3H]Valine (23 Ci/mmol), with 5 μM tRNA substrate and 20 μM T222P IleRS. Mischarging reactions were incubated at room temperature for 30–60 min and worked up as described above.

The same strategy was used to prepare mischarged tRNAval and the respective 3'-end variants. The T222P mutant of E. coli ValRS was found to effectively mischarge wild-type and other tRNAval derivatives with threonine (28). The conditions for mischarging were identical to those for the T222P IleRS mischarging of tRNAval, except that 8 μM [3H]Threonine (50 Ci/mmol) replaced valine. T222P ValRS was used at a concentration of 5 μM, as was the tRNA substrate. The yield of mischarged product depended on the incubation time more critically than seen with tRNAval mischarging reactions, likely due to a low level of editing activity with T222P ValRS. Optimal yields were achieved using a 5-min incubation for mischarging wild-type tRNAval, a 20–30-min incubation with 3'-dA76 tRNAval, and a 1-h incubation with 3'-fluoro-A76 tRNAval. All reactions were performed at room temperature and worked up as described above.

**Decaylation Assays—**Decaylation was also measured using the tri-chloroacetic acid precipitation technique. Decaylation of mischarged mature tRNAval was done at room temperature in 150 mM Tris-HCl (pH 7.5), 20 mM MgCl2, using 1 μM mischarged tRNAval. For decaylation reactions of the decayed tRNAval, ΔA76 tRNAval with other tRNAs, tRNAval transcripts were used. Not only were transcripts free of tRNAval contamination, but the 3'-end-modified derivatives also had less contamination of their 3'-ends with residual A76 because the exchange reactions utilized ΔA76 tRNAval as the source tRNA (generally 99.5% exchange was achieved as opposed to 98–99%). The decaylation assays using these mischarged transcript-derived substrates

2 C.-C. Wang, unpublished data.
To further investigate the role of the 3′-OH group of tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}}} (22) as the initial site of aminoacylation, a feature now known to be determined by the 2′-OH group in reactions with altered 3′-OH variants. Early work established that IleRS and ValRS utilize the 2′-OH group of tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}}} for proper aminoacylation by IleRS and thus, the 2′-OH group is important for deacylation activity. Replacement of the 3′-OH group with either a hydrogen or fluorine atom. While the plots in Fig. 2A show a slow deacylation rate for Val-3′-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} and Val-3′-fluoro-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{i}}}}}}, these conditions were met to identify the relative charging plateaus (isoleucine versus valine) for 3′-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} and 3′-fluoro-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} substrates. Aminoacylation of tRNA\textsubscript{\textit{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} has been shown to be critical for editing, and lack of editing manifests itself in 3′-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} being completely mischarged with valine by IleRS. This mischarging is in striking contrast to the cis-diol containing A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}}, which is not detectably mischarged with valine.

To more closely examine the properties of the 3′-OH group of tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} that are critical for editing, a 3′-fluoro-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} was constructed. Fluorine better emulates the electronegative properties of the OH group and may even serve as a hydrogen bond acceptor (38–40). If these were the properties of the OH group that promoted editing, then 3′-fluoro-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} should be difficult to mischarge. However, IleRS rapidly mischarged 3′-fluoro-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} with valine (Fig. 1A). As expected, we observed no charging or mischarging with 2′-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} or 2′-fluoro-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}}.

It is worth noting that the data in Fig. 1 also demonstrate the purity of the tRNA products isolated from the tRNA N7ase exchange reactions. As the relative charging plateaus (isoleucine versus valine) for 3′-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} and 3′-fluoro-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} are equal for both tRNA substrates, it is likely that virtually no wild-type tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} (which only charges with isoleucine) remains in the preparations. The same conclusion can be drawn from the observation that neither 2′-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} nor 2′-fluoro-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} display any charging activity.

The Terminal 3′-OH of tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} Is Important for Decaylation of Mischarged tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}}.—IleRS has a potent deacylation activity toward mischarged A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} (Fig. 2A). This activity is completely abolished by the replacement of the terminal 3′-OH with either a hydrogen or fluorine atom. (While the plots in Fig. 2A show a slow deacylation rate for Val-3′-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} and Val-3′-fluoro-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} under these conditions the rates were identical to the rates in the absence of enzyme.) Under conditions designed to promote rapid deacylation rates (4 μM mischarged tRNA substrate, 1 μM IleRS), the complete lack of deacylation activity toward Val-3′-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} is striking (Fig. 2B). The A\textsubscript{76} tRNA was completely deacylated in 2–3 min, whereas the 2′-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} was only about 3% deacylated after 1 h. This amounts to a more than 750-fold decrease in deacylation rate.

IleRS Deacylates Mischarged 2′-A\textsubscript{76} Substrates.—The results with 3′-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} and 3′-fluoro-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} confirm and expand upon previous work detailing the importance of the 3′-OH group in hydrolytic editing. The question still remains to what is the mechanistic reason behind the importance of the 3′-OH. One possibility is that the 3′-OH serves a catalytic function to facilitate deacylation from the 2′-OH. Alternatively, the mischarged amino acid may be esterified to the 3′-OH just prior to hydrolysis. The well-characterized 2′- to 3′-transacylation reaction on the cis-diol-containing wild-type tRNA could reposition the amino acid. A similar transacylation is not possible for either 3′-A\textsubscript{76} or 3′-fluoro-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}}. If transacylation were a prerequisite to deacylation, then a mischarged 2′-A\textsubscript{76} tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} (containing a 3′-aminoacyl linkage) might be expected to be a substrate for deacylation.

To investigate this possibility we had to develop a method for producing mischarged 2′-A\textsubscript{76} substrates. We attempted to mischarge 2′-A\textsubscript{76} substrates using a number of different aminoacyl-tRNA synthetases and reaction conditions. For this purpose, we used both transcripts (as opposed to the mature, cell-isolated tRNA used in the previous experiments) of tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}} and RNA minihelices based on the acceptor-T\textsubscript{\textit{\textit{\textit{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}}}} stem of tRNA\textsuperscript{\textit{\textit{\textit{\textit{\textit{\textit{i}}}}}}}. Most mischarging trial reactions were unsuccessful. Nei-
ther editing-deficient mutants of IleRS nor *E. coli* or *Bacillus stearothermophilus* ValRS had any charging activity with 2'-dA76 substrates. The highest degree of mischarging was achieved using ValRS from yeast. While the reactions were not efficient, about 6–8% of the input 2'-dA76 tRNA^{Ile} and 1% of 2'-dA76 minihelix^{Ile} was mischarged in incubations using yeast ValRS in the presence of 30% dimethyl sulfoxide (Me_2SO) and no monovalent cations. The T379A,T380A double mutant yeast ValRS gave identical yields and was used interchangeably with wild-type yeast ValRS (see “Experimental Procedures”). Because the fraction of RNA that was successfully mischarged in these preparations was low, contamination of starting materials with wild-type (non-exchanged) RNAs was investigated. The 2'-dA76 tRNA^{Ile} used in these reactions was derived from ΔA76 tRNA^{Ile} transcripts. This starting material has the advantage of being free of any tRNA^{Val} species (that might be recognized by ValRS) and of having the lowest possible contamination with the cis-diol containing A76 tRNA^{Ile} that can arise from incomplete exchange of the A76 terminus. Under standard aminoacylation conditions with isoleucine, 2'-dA76 tRNA^{Ile} transcripts showed about 0.5% of the charging level that would be expected based on its absorbance at 260 nm. However, the large excess of 2'-dA76 tRNA^{Ile} in these preparations could be inhibiting the aminoacylation of A76 tRNA^{Ile} and thus masking the amount of A76 tRNA^{Ile} present. To rule out this possibility, a control compared the level of aminoacylation of 2'-dA76 tRNA^{Ile} alone to that of 2'-dA76 tRNA^{Ile} intentionally spiked with 3% A76 tRNA^{Ile}. The reaction containing the 3% A76 tRNA^{Ile} showed complete charging in 10 min (data not shown), while the 2'-dA76 tRNA^{Ile} alone showed almost no charging. Thus, the 6–8% yield of mischarged 2'-dA76 tRNA^{Ile} cannot be due to contamination with A76 tRNA^{Ile}. A similar experiment showed that 2'-dA76 minihelix^{Ile} was free of wild-type minihelix^{Ile}. This result was expected, as the starting material for chemical RNA synthesis is homogenous.

These mischarged 2'-dA76 substrates were tested in deacylation assays relative to their A76 and 3'-dA76 variants. Both Val-2'-dA76 tRNA^{Ile} and Val-2'-dA76 minihelix^{Ile} were efficiently deacylated by IleRS. Relative to Val-A76 tRNA^{Ile}, the 2'-dA76 derivative showed an approximate 5-fold decrease in the initial rate of deacylation (Fig. 4A). This decrease is in striking contrast to the 750-fold or more drop in deacylation rate observed with Val-3'-dA76 tRNA^{Ile}. The hydroxyl specificity of deacylation was maintained with regards to the minihelix, as a Val-3'-dA76 minihelix^{Ile} was completely resistant to hydrolysis (Fig. 4B). No difference in rate was detected between the deacylation rates for Val-minihelix^{Ile} and Val-2'-dA76 minihelix^{Ile}.

The 3'-OH of tRNA^{Val} Is Important for Preventing Misacylation by ValRS—To compare how editing specificity may have adapted through evolution, we performed analogous experiments with the closely related ValRS. While, no misacylation of A76 tRNA^{Val} was detected in any of the conditions tested, substitution of the 3'-OH group of tRNA^{Val} with either a hydrogen or fluorine atom gave a tRNA that is mischarged with threonine (Fig 5A). Additionally, these modifications had little effect on the aminoacylation activity with valine (Fig. 5B). Under the assay conditions used (Fig. 5, A and B), the plateau level of charging of 3'-dA76 tRNA^{Val} with threonine was significantly lower than with valine. When aminoacylation reactions were performed with higher concentrations of ValRS, the charging plateaus with valine and threonine were identical for
3′-dA76 tRNAVal (Fig. 5). (Thus, the nucleotide exchange reactions with 3′-dATP gave essentially pure 3′-dA76 tRNAVal, and the observed intermediate threonine mischarging plateau (Fig. 5A) cannot be due to contamination with A76 tRNAVal.) Mischarging of 3′-fluoro-A76 tRNAVal with threonine was complete even under the conditions of a lower ValRS/tRNA ratio than needed for complete mischarging of 3′-dA76 tRNAVal (Fig. 5A).

ValRS Does Not Require an Intact 3′-OH to Edit Mischarged tRNA—In contrast to IleRS, a mischarged 3′-dA76 substrate is a substrate for deacylation (Fig. 6A). (A similar finding was reported for yeast ValRS (19).) This modification decreases the editing efficiency of both IleRS and ValRS, yet the observed ValRS catalyzed deacylation of Thr-3′-dA76 tRNAVal demonstrates a marked difference in substrate specificity for editing. The mischarging with threonine of 3′-dA76 tRNAVal is the result of competing aminoacylation and deacylation reactions (Fig. 5A). Because the deacylation activity is reduced it cannot keep pace with the mischarging.

To further pursue investigation of 3′-modified tRNAVal substrates, Thr-3′-fluoro-A76 tRNAVal was constructed. Little deacylation of Thr-3′-fluoro-A76 tRNAVal was detected over the relatively high rate of non-enzyme catalyzed deacylation (Fig. 6A). This result is consistent with the full mischarging of 3′-fluoro-A76 tRNAVal under conditions where 3′-dA76 tRNAVal is only partially mischarged. With higher concentrations of ValRS, Thr-3′-fluoro-A76 tRNAVal is indeed deacylated by ValRS (Fig 6B). Thus, ValRS has distinct substrate specificity relative to its close homolog IleRS. Whether ValRS simply has broader hydroxyl specificity or has reversed hydroxyl specificity relative to IleRS is unanswered. Attempts to mischarge 2′-dA76
tRNAVal by a variety of approaches and subsequently measure deacylation of Thr-2O-Acyl 
linkages—The results with ValRS raised the possibility that editing is inherently "plastic" and that, given the appropriate stimulus, IleRS might also deacylate substrates mischarged at the 2'-OH. Here, the minihelix system was of particular advantage relative to the use of tRNA substrates. A 3'-O-Me-A76 minihelixVal (3'-OMe-A76) could be synthesized from commercially available starting materials, whereas 3'-OMe-ATP is not a substrate for tRNA NTase. A mischarged 3'-OMe-A76 minihelixVal has a methyl group that prevents a 2'-O to 3'-transacylation from occurring. Although this molecule has an intact 2'-OH, it was not aminoacylated by IleRS. Successful aminoacylation was only achieved using yeast ValRS under the same conditions (30% MeSO and no monovalent cations) that were used for mischarging 2'-dA76 substrates.

Interestingly, Val-3'-OMe-A76 minihelixVal was efficiently deacylated by IleRS (Fig. 7A). To verify that this deacylation activity was catalyzed by the editing site in CP1, we demonstrated that the editing-deficient mutant T242P IleRS was unable to deacylate Val-3'-OMe-A76 minihelixVal (data not shown). (The T242P mutation is located in the editing pocket of CP1 (12).) The rate of Val-3'-OMe-A76 minihelixVal deacylation is only 2-fold reduced from that of the wild-type or 2'-dA76 minihelix. B, with 20% methanol added to the solvent, even a mischarged 3'-dA76 tRNA can be deacylated. Under these conditions the molecular recognition in the editing active site is disrupted to the point where properly charged 3'-dA76 tRNAVal is also deacylated.

**DISCUSSION**

A mischarged 3'-dA76 tRNAVal is completely resistant to deacylation by IleRS. This resistance is most likely because the
aminoacyl linkage prevents the scissile ester bond from coming into close proximity of the hydrolytic machinery when bound in the editing site. The magnitude of the rate difference (≥750-fold) between deacylation of Val-2'-dA76 tRNAVal and Val-3'-dA76 tRNAVal is larger than one might expect if the 3'-OH had a noncovalent role in deacylation. The calculated difference in transition state stabilization of ~4 kcal/mol is beyond the range of energies observed for average hydrogen bonds or the electro-negative inductive effect of a neighboring hydroxyl. Correspondingly, a fluoro group in place of the 3'-OH failed to allow for even partial deacylation activity. Earlier work did not directly investigate the deacylation of Val-2'-dA76 tRNAVal and, given the available data, concluded that the role of the 3'-OH was to assist catalysis of deacylation from the 2'-OH (43). Here, reasonable quantities of mischarged 2'-dA76 tRNAVal were produced, isolated, and directly shown to be deacylated by IleRS.

This mischarged substrate, with a fixed 3'-O-aminoacyl linkage, showed only a modest 5-fold decrease in initial rate of deacylation. This rate decrease is in the range of what might be expected for the loss of neighboring group effects due to the missing 2'-OH (20). Thus, under normal circumstances, transacylation from the 2'- to the 3'-OH is required for deacylation of Val-tRNAVal by IleRS.

Although IleRS cannot deacylate Val-3'-dA76 tRNAVal, VaRS deacylates Thr-3'-dA76 tRNAVal with an approximate 10-fold reduction in rate compared with deacylation of Thr-76 tRNAVal. Still, it is possible that VaRS preferentially deacylates 3'-O-aminoacyl esters. Even though the initial site of aminoacylation is the 2'-OH, because transacylation is more rapid than deacylation (21), a 3'-O-aminoacyl ester could be the main substrate for editing. In that event, a 10-fold reduced deacylation rate of Thr-3'-dA76 tRNAVal relative to Thr-76 tRNAVal would not be surprising.

Alternatively, the role of the 3'-OH of tRNAVal may be as a hydrogen bond donor (Fig. 8). In this model, derived from studies of non-catalyzed ester hydrolysis by Bruice and co-workers (20), the neighboring hydroxyl donates a hydrogen to the oxanion of the tetrahedral intermediate, thereby helping to stabilize the build up of negative charge (Fig. 8, left). The 3'-daA analog is obviously unable to fulfill this role (Fig. 8, middle). Not only is the fluoro group unable to donate a hydrogen bond, but its partial negative charge likely also induces some electrostatic repulsion that distorts the tetrahedral intermediate (Fig. 8, right). This model fits best with the observed trends in deacylation rates for these substrates.

The observed deacylation rate of Val-3'-OmeA76 5minihelix is by IleRS demonstrates a shift in substrate specificity for deacylation reminiscent of the substrate specificity VaRS shows. The mechanism by which IleRS is able to hydrolyze this 2'-O-aminoacyl ester likely arises from some local structural rearrangement induced by the bulkier 3'-end as opposed to a hydrogen bonding role for the 3'-OMe group (the 3'-OH cannot play a similar hydrogen-bonding role during deacylation of Val-2'-dA76 substrates). In the context of a 2'-O-aminoacyl ester, the editing active site may be too crowded to accommodate the 3'-O-Me group in the normal binding orientation. Alternatively, there could be a small hydrophobic interaction that is made with the 3'-O-Me group, also altering the position of the scissile ester bond with respect to the catalytic center. Such local structural perturbations are certainly feasible within the IleRS-editing site, as evidenced by the deacylation activity seen in the presence of 20% methanol.

Regardless of the detailed mechanism, the results show that the editing site is inherently plastic with respect to recognition of mischarged tRNA. For IleRS and VaRS to discriminate against valine and threonine, respectively, the editing sites of both enzymes must recognize subtle differences in aminoacyl side chains. Yet this fine structure discrimination must also be flexible, as both enzymes have been shown to edit multiple non-cognate amino acids (44). A third, related synthetase, leucyl-tRNA synthetase, has also been reported to edit multiple amino acids (45, 46). This remarkable combination of specificity and plasticity in recognition of the aminoacyl side chain is shown here to include the position of the aminoacyl group on the 3'-end of tRNA. This plasticity may have developed over a long period of evolution and may have been particularly important in the early stages of the development of aminoacylation systems and the genetic code, when aminoacyl-RNA substrates were not perfected. Indeed, the CP1 insertion is ancient, as it is conserved throughout evolution and found in deeply rooted organisms of all three kingdoms such as the Thermogales, Crenarchaeota, and Diplomonads (47).

Acknowledgments—We thank Dr. Nancy Maiels and Dr. Alan Weiner for providing the plasmid encoding tRNA NTase, and Dr. Chien-Chia Wang for cloning and expressing yeast VaRS. We are grateful to Dr. T. Hendrickson, Dr. T. Nomanbhoy, L. Nangle, and Dr. M. Sprinzl for help in preparing materials and insightful discussion.

REFERENCES
1. Carter, C. W., Jr. (1993) Annu. Rev. Biochem. 62, 715–748
2. Giege, R., Puglisi, J. D., and Florentz, C. (1993) Prog. Nucleic Acids Res. Mol. Biol. 45, 129–206
3. Pauling, L. (1956) Festschrift fuer Prof. Dr. Arthur Stoll, Birkhäuser Verlag, Basel, Switzerland
4. Schmidt, E., and Schimmel, P. (1994) Science 264, 265–267
5. Löffler, R. B. (1963) Biochem. J. 89, 82–92
6. Baldwin, A. N., and Berg, P. (1964) J. Biol. Chem. 241, 819–845
7. Eldred, E. W., and Schimmel, P. R. (1972) J. Biol. Chem. 247, 2961–2964
8. Fersht, A. R., and Dingwall, C. (1979) Biochemistry 18, 2627–2631
9. Fersht, A. R., and Kaelin, M. M. (1976) Biochemistry 15, 3342–3346
10. Hale, S. P., Auld, D. S., Schmidt, E., and Schimmel, P. (1997) Science 276, 1250–1252
11. Lin, H., Hale, S. P., and Schimmel, P. (1998) Nature 394, 53–54
12. Nureki, O., Vaasulyev, D. G., Tateno, M., Shimada, A., Nakama, T., Fukui, S., Konno, M., Hendrickson, T. L., Schimmel, P., and Yokoyama, S. (1998) Science 280, 578–582
13. Silvan, L. F., Wang, J., and Steitz, T. A. (1999) Science 285, 1074–1077
14. Fukui, S., Nureki, O., Sekine, S., Shimada, A., Tao, J., Vassulyev, D. G., and Yokoyama, S. (2000) Cell 103, 793–803
15. Schmidt, E., and Schimmel, P. (1995) Biochemistry 34, 11204–11210
16. Hendrickson, T. L., Nomanbhoy, T. K., and Schimmel, P. (2000) Biochemistry 39, 8180–8186
17. von der Haar, F., and Cramer, F. (1975) FEBS Lett. 56, 215–217
18. von der Haar, F., and Cramer, F. (1975) Biochemistry 14, 4131–4138
19. Igloi, G. L., von der Haar, F., and Cramer, F. (1977) Biochemistry 16, 1696–1702
20. Bruice, T. C., and Fife, T. H. (1962) J. Am. Chem. Soc. 84, 1973–1979
21. Tatlil, M., Yokoyama, S., and Miyazawa, T. (1983) Biochemistry 22, 3220–3225
22. Cramer, F., Faulhammer, H., von der Haar, F., Sprinzl, M., and Sternbach, H. (1975) FEBS Lett. 56, 212–214
23. Shibata, K., and Schimmel, P. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1880–1884
24. Hendrickson, T. L., Nomanbhoy, T. K., de Crécy-Lagard, V., Fukui, S., Nureki, O., Yokoyama, S., and Schimmel, P. (2002) Mol. Cell 9, 353–362
25. Sheppard, A., Shibata, K., and Schimmel, P. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9964–9968
26. Fersht, A. R., Ashford, J. S., Bruton, C. J., Jakes, R., Koch, G. L., and Hartley, B. S. (1975) Biochemistry 14, 1–4
27. Nomanbhoy, T. K., and Schimmel, P. R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5119–5122
28. Doring, V., Most, H. D., Nangle, L. A., Hendrickson, T. L., de Crécy-Lagard,
Recognition of Mischarged tRNA

20517

V., Schimmel, P., and Marliere, P. (2001) Science 292, 501–504
29. Shi, P. Y., Weiner, A. M., and Maizels, N. (1998) RNA 4, 276–284
30. Schmidt, E. (1996) Amino Acid Recognition by a Class I tRNA Synthetase. Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA, p. 160
31. Glasfeld, E., Landro, J. A., and Schimmel, P. (1996) Biochemistry 35, 4139–4145
32. Farrow, M. A., Nordin, B. E., and Schimmel, P. (1999) Biochemistry 38, 16898–16903
33. Milligan, J. F., and Uhlenbeck, O. C. (1989) Methods Enzymol. 180, 51–62
34. Wincott, F., DiRenzo, A., Shaffer, C., Grimm, S., Tracz, D., Workman, C., Sweedler, D., Gonzalez, C., Searinge, S., and Usman, N. (1995) Nucleic Acids Res. 23, 2677–2684
35. Francis, T. A., Ehrenfeld, G. M., Gregory, M. R., and Hecht, S. M. (1983) J. Biol. Chem. 258, 4279–4284
36. Sprinzl, M., and Cramer, F. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 3049–3053
37. Fraser, T. H., and Rich, A. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 3044–3048
38. Pyle, A. M., Murphy, F. L., and Cech, T. R. (1992) Nature 358, 123–128
39. Yoshizawa, S., Fourmy, D., and Puglisi, J. D. (1999) Science 285, 1722–1725
40. Burke, T. R., Ye, B., Yan, X., Wang, S., Jia, Z., Chen, L., Zhang, Z. Y., and Barford, D. (1996) Biochemistry 35, 15989–15996
41. Nureki, O., Niimi, T., Muto, Y., Kanno, H., Kolno, T., Muramatsu, T., Kawai, G., Miyazawa, T., Giegé, R., Florentz, C., and Yokoyama, S. (1993) in The Translational Apparatus (Nierhaus, K. H., Franceschi, F., Subramanian, A. R., Erdmann, V. A., and Wittman-Liebold, B., eds) pp. 59–66, Plenum Press, New York
42. Alexander, R. W., Nordin, B. E., and Schimmel, P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12214–12219
43. Freist, W., and Cramer, F. (1983) Eur. J. Biochem. 131, 65–80
44. Jakubowski, H., and Goldman, E. (1992) Microbiol. Rev. 56, 412–429
45. Englisch, S., Englisch, U., von der Haar, F., and Cramer, F. (1986) Nucleic Acids Res. 14, 7529–7539
46. Mursinna, R. S., Linecum, T. L., and Martinis, S. A. (2001) Biochemistry 40, 5376–5381
47. Schimmel, P., and Ribas de Pouplana, L. (2002) Cold Spring Harbor Symp. Quant. Biol., in press
48. Nordin, B. E., and Schimmel, P. (1999) J. Biol. Chem. 274, 6835–6838