Enzyme Hyperspecificity. Rejection of Threonine by the Valyl-tRNA Synthetase by Misacylation and Hydrolytic Editing†

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ABSTRACT: Valyl-tRNA synthetase from *Bacillus stearothermophilus* activates threonine and forms a 1:1 complex with threonyl adenylate, but it does not catalyze the net formation of threonyl-tRNA^{Val}. On mixing the enzyme-bound threonyl adenylate complex with tRNA^{Val} at pH 7.78 and 25 °C in the quenched flow apparatus it decomposes at a rate constant of 36 s^{-1}. During this process there is a transient formation of Thr-tRNA^{Val} reaching a maximum at 25 ms and rapidly falling to zero after 150 ms. At the peak, 22% of the [^{14}C]threonine from the complex is present as [^{14}C]Thr-tRNA. The reaction may be quenched with phenol and the partially mischarged tRNA isolated. The enzyme catalyzes its hydrolysis with a rate constant of 40 s^{-1}. The data fit a kinetic scheme in which 62% of the threonine from the threonyl adenylate is transferred to the tRNA. This may be compared with the rate constant of 12 s^{-1} at which 84% of the valine is transferred to tRNA^{Val} from the enzyme-bound valyl adenylate, and the rate constant of 0.015 s^{-1} for the subsequent hydrolysis of Val-tRNA^{Val}. Inhibition studies indicate a distinct second site for hydrolysis. The translocation of the aminoacyl moiety between the two sites could be mediated by a transfer between the 2′- and 3′-OH groups of the terminal adenosine of the tRNA. The hyperspecificity of the enzyme is based on discriminating between the two competing substrates twice: once against the undesired substrate in the synthetic step, and once against the desired substrate in the destructive step.

During protein biosynthesis the cell distinguishes between certain amino acids with a specificity far greater than that expected from the differences in their structures. For example, as valine is one methylene group smaller than isoleucine it binds to the isoleucyl-tRNA synthetase (IRS), although 100 times more weakly than isoleucine (Flossdorf and Kula, 1973). Yet, it has been found that the overall error rate in protein biosynthesis is less than 1 part in 3000 (Loftfield, 1963; Loftfield and Vanderjagt, 1972). The origin of the increased specificity was suggested by Crick (see Crick, 1975) and found experimentally by (Norris) Baldwin and Berg, to be due to an editing hydrolytic reaction (Norris and Berg, 1964; Baldwin and Berg, 1966). The aminoacylation reaction takes place in two steps (eq 1): the activation of the amino acid by the formation of an aminoacyl adenylate complex followed by the transfer of the aminoacyl moiety to its cognate tRNA (Berg, 1961; Fersht and Kaethner, 1976). Isoleucyl-tRNA synthetase catalyzes the first step with valine and ATP

$$\mathrm{IRS}(\pm\mathrm{tRNA}) \xrightarrow[\mathrm{Ile}]{\mathrm{ATP}} \mathrm{IRS}\cdot\mathrm{Ile}{\sim}\mathrm{AMP}\ (\pm\mathrm{tRNA}) + \mathrm{PP_i}$$

$$\mathrm{IRS}\cdot\mathrm{Ile}{\sim}\mathrm{AMP}\cdot\mathrm{tRNA} \rightarrow \mathrm{IRS} + \mathrm{Ile\text{-}tRNA} + \mathrm{AMP} \quad (1)$$

but, whereas the addition of tRNA^{Ile} to the isolated IRS·Ile~AMP complex leads to the transfer of about 70% of the isoleucine to the tRNA, the addition of tRNA^{Ile} to the IRS·Val~AMP complex causes its quantitative hydrolysis (Baldwin and Berg, 1966). The net result is that in the presence of tRNA^{Ile} and valine, the IRS acts as an ATP pyrophosphatase, converting ATP to AMP.

The exact mechanism of this editing step is unclear. It has been suggested that the tRNA is first misacylated and the mischarged tRNA specifically hydrolyzed by the enzyme (Yarus, 1972; Eldred and Schimmel, 1972). In support of this it was shown that the aminoacyl-tRNA synthetases may slowly hydrolyze their correctly aminoacylated tRNAs, and in some cases rapidly hydrolyze artificially misacylated tRNAs. However, it has been maintained that, in general, these hydrolysis rates are too low to account for the observed specificities (Bonnet and Ebel, 1974), and that the hydrolysis is nonspecific (Bonnet, 1974; Sourgoutchoff et al., 1974).

We wish to present direct evidence from rapid quenching experiments that the valyl-tRNA synthetase (VRS) from *Bacillus stearothermophilus* discriminates against threonine, which is isosteric with valine, by first mischarging tRNA^{Val} with threonine and then correcting the error by enzymatically hydrolyzing the Thr-tRNA^{Val}.

Materials and Methods

The VRS from *B. stearothermophilus* was as previously described (Fersht et al., 1975; Fersht, 1975).

tRNA from *B. stearothermophilus* was obtained from Professor B. S. Hartley, Imperial College of Science and Technology, London. It was initially partially fractionated on benzoylated diethylaminocellulose (eluting NaCl, from 0.4 to 1.0 M, and from 1.0 M to 1.0 M + 20% ethanol, all with 10 mM $MgCl_2$) to obtain tRNA$_1^{Val}$ (valyl acceptance = 190 pmol/A_{260}) and tRNA$_2^{Val}$ (valyl acceptance = 100 pmol/A_{260}). Purification of tRNA$_1^{Val}$ to near homogeneity (valyl acceptance = 1400 pmol/A_{260}) was accomplished by chromatography on DEAE-Sephadex (A-50, eluting with a gradient of 0.375 M NaCl, 8 mM $MgCl_2$–0.525 M NaCl, 16 mM $MgCl_2$; 20 mM Tris-Cl, pH 7.5) followed by the reverse salt gradient procedure with Sepharose 4B (Holmes et al., 1975).

Radioactively labeled amino acids were obtained from The Radiochemical Centre, Amersham, England. The radiochemical purity of the [^{14}C]Thr (batch 16) was quoted at 97–99%. High-voltage electrophoresis at pH 2.1 on Whatman

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‡ Abbreviations used are: IRS, isoleucyl-tRNA synthetase; VRS, valyl-tRNA synthetase; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; ATP, adenosine 5′-triphosphate.

No. 4 paper showed that 98% of the radioactivity moved with the threonine and less than 0.5% could be associated with valine.

Experiments were performed in buffers containing 10 mM mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride, 10 mM $MgCl_2$, and either 144 mM Tris-Cl (pH 7.78 at 25 °C) or 13 mM Bistris-Cl (pH 5.87 at 25 °C, pH 6.28 at 0 °C). The exchange of [^{32}P]pyrophosphate (2 mM) into ATP (2 mM) at pH 7.78 was measured by the adsorption of ATP on charcoal. The rate of aminoacylation of tRNA was determined by the precipitation of the resultant [^{14}C]Val-tRNA by trichloroacetic acid, collection on nitrocellulose or glass fiber filters. After washing copiously with further trichloroacetic acid (containing valine) and drying, the radioactivity was monitored by scintillation spectrophotometry using a toluene-based scintillant.

The threonine stimulated ATP pyrophosphatase activity was measured from the release of [^{32}P]pyrophosphate from [γ-^{32}P]ATP, the ATP being adsorbed on charcoal and separated by centrifugation (Baldwin and Berg, 1966; Fersht and Kaethner, 1976).

Alkaline phosphatase (*E. coli*) was routinely added where necessary to destroy AMP.

tRNA Stimulated Hydrolysis of VRS·Thr~[^{32}P]AMP. VRS·Thr~[^{32}P]AMP was prepared from VRS, threonine, [α-^{32}P]ATP, and inorganic pyrophosphatase, and isolated by gel filtration at 0 °C and pH 6.28 as described for VRS·[^{14}C]Val~AMP (Fersht, 1975). The rate of hydrolysis of the complex at 0 °C, pH 6.28, was followed by the method of Baldwin and Berg (1966), by monitoring the alkaline phosphate catalyzed release of [^{32}P]orthophosphate from the [^{32}P]AMP that is released. tRNA (20–30 A_{260} units) was added to a solution of the adenylate (200 μl, 4 μM) containing 0.6 unit/ml of alkaline phosphatase.

Transfer of [^{14}C]Thr to tRNA^{Val} at 0 °C. VRS·[^{14}C]-Thr~AMP was prepared at pH 6.28 from [^{14}C]Thr (232 mCi/mmol), ATP, and inorganic pyrophosphatase, as described for VRS·[^{14}C]Val~AMP (Fersht, 1975). tRNA was added to a solution of the adenylate (1–4 μM) and alkaline phosphatase (0.6 unit/ml), samples were rapidly quenched with trichloroacetic acid, and the precipitates were collected on nitrocellulose filters.

Preparative Mischarging of tRNA^{Val} with [^{14}C]Thr. tRNA^{Val} (150 μl, 6.4 nmol) was added at 0 °C to a solution of VRS·[^{14}C]Thr~AMP (1.5 ml, 6.4 nmol) at pH 6.28 and immediately quenched by the addition of a saturated solution of PheOH and chloroform. After centrifugation and precipitation of the tRNA from the aqueous layer (0.2 M sodium acetate, pH 5.5) by the addition of two volumes of ethanol, the tRNA was dissolved in 10 mM $MgCl_2$ (pH 5) and freed from ethanol by gel filtration (10 mM $MgCl_2$, Sephadex G-25), to give 2.6 pmol of tRNA^{Val} that had been 3.8% aminoacylated.

Rapid Quenching Experiments at 25 °C and pH 7.78. (a) Hydrolysis of [^{14}C]Thr-tRNA^{Val}. One syringe of the quenched flow apparatus (Fersht and Jakes, 1975) contained VRS (2 μM) incubated in 288 mM Tris-Cl (pH 7.78), 10 mM $MgCl_2$, 10 mM mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride. The other contained tRNA^{Val} (320 nM) and [^{14}C]Thr-tRNA^{Val} (12 nM) unbuffered in 10 mM $MgCl_2$. The solutions were automatically mixed and quenched with trichloroacetic acid (5%), either 123 or 188 μl being expelled from each syringe.

(b) Hydrolysis of VRS·Thr~[^{32}P] AMP. One syringe contained tRNA^{Val} (34 μM) in 288 mM Tris-Cl as above, and the other, VRS·Thr–[^{32}P]AMP (1.86 μM) in 13 mM Bistris-Cl (pH 5.87). The solutions were mixed and quenched with perchloric acid (3.5%) as described above. Aliquots of the effluent were immediately added to Tris-Cl (1.0 M Tris, 0.44 M Tris–HCl, 0 °C) containing alkaline phosphatase (10 units/ml) and quenched with perchloric acid after 20.0 s.

The [^{32}P]orthophosphate released from the [^{32}P]AMP was monitored as described by Baldwin and Berg, 1966.

(c) Transient Formation of [^{14}C]Thr-tRNA. One syringe of the apparatus contained VRS·[^{14}C]Thr~AMP (1.8 μM) and alkaline phosphatase (0.03 unit/ml) in Bistris-Cl (pH 5.87), the other tRNA^{Val} (34 μM) in Tris-Cl as above. The solutions were mixed and quenched with trichloroacetic acid, and the precipitates collected on nitrocellulose filters.

For b and c the rate of hydrolysis of the VRS·Thr~AMP complex was monitored periodically by filtering aliquots through nitrocellulose filters (Yarus and Berg, 1970). During the course of the experiments less than 20% hydrolyzed ($t_{1/2}$ = 3 h).

Transfer of [^{14}C]Val to tRNA^{Val}. One syringe of the quenched flow apparatus contained VRS·[^{14}C]Val~AMP (0.26 μM) in Bistris-Cl, the other tRNA^{Val} (11 μM) in the concentrated Tris-Cl. Aliquots were periodically quenched with trichloroacetic acid.

Hydrolysis of [^{14}C]Val-tRNA^{Val}. [^{14}C]Val-tRNA^{Val} was prepared in situ by adding tRNA^{Val} (0.5 μM) to a solution of VRS·[^{14}C]Val~AMP (0.11 μM), VRS (4 or 2 μM), and alkaline phosphatase (0.7 unit/ml) at 25 °C, and pH 7.78. Aliquots were quenched at 15-s intervals with trichloroacetic acid.

TABLE I: Comparison of Pyrophosphate Exchange, Aminoacylation, and ATP Pyrophosphase Activities of VRS.[a]

Reaction	Amino Acid	k_{cat} s^{-1}	K_M mM
Pyrophosphate[b,c] exchange	Val	33	0.03
	Thr	14	7
Pyrophosphate exchange in presence of tRNA[b,c,d]	Val	33	–
	Thr	10	–
Aminoacylation[e]	Val	3	–
ATP pyrophosphatase[c]	Thr	6	7

[a] 25 °C, pH 7.78, 10 mM $MgCl_2$, 2 mM ATP. [b] 2 mM pyrophosphate. [c] 185 nM VRS. [d] 8.6 μM tRNA^{Val}, 37 nM VRS.

Results

The VRS from *B. stearothermophilus,* like its counterpart from *E. coli* (Bergmann et al., 1961; Yaniv and Gros, 1969; Owens and Bell, 1970), catalyzes the exchange of [^{32}P]pyrophosphate into ATP in the presence of threonine, as well as valine (Table I), although k_{cat} is some 2.4 times lower and K_m 200 times higher. This exchange is not due to impurities in the threonine. Incubation of the enzyme with ATP, inorganic pyrophosphatase, and a fourfold excess of [^{14}C]Thr leads to the formation and isolation by gel filtration of a VRS·[^{14}C]-Thr~AMP complex with a stoichiometry of 1.0. Since the [^{14}C]Thr was found to be at least 98% pure, this result cannot be due to a contamination of [^{14}C]Val.

Incubation of the VRS with tRNA, [^{14}C]Thr, ATP, and inorganic pyrophosphatase gives no detectable formation of [^{14}C]Thr-tRNA under a wide variety of conditions, from pH 5.9 to 7.8, and 0 to 75 °C. Instead, the enzyme functions as an ATP pyrophosphatase. The turnover number for this ap-

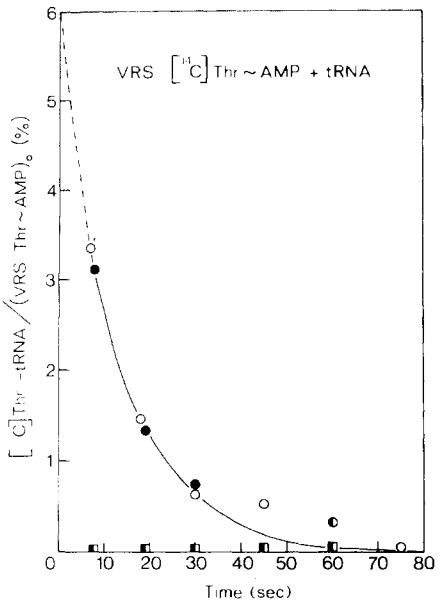

FIGURE 1: Transient formation of Thr-tRNA at 0 °C, pH 6.28. tRNA (10 nmol) was added to a solution of VRS·[^{14}C]Thr~AMP (4 μM, 220 μl) and alkaline phosphatase (0.6 unit/ml) in a buffer containing 10 mM Bistris-Cl, 10 mM $MgCl_2$, and 10 mM mercaptoethanol. (○) $tRNA_1^{Val}$ (0.9 nmol); (●) $tRNA_2^{Val}$ (0.9 nmol); (□) tRNA fractions enriched for either $tRNA^{Phe}$ or $tRNA^{Tyr}$ ($tRNA^{Val}$ < 5 pmol). The ratio of Thr-tRNA to initial concentration of VRS·[^{14}C] Thr~AMP is plotted. The curve is for a rate constant of 0.077 s^{-1}.

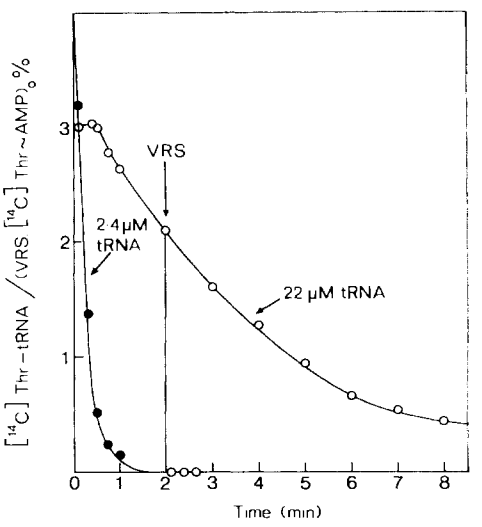

FIGURE 2: Competitive inhibition by $tRNA^{Val}$ of the hydrolysis of Thr-$tRNA^{Val}$ at 0 °C and pH 6.28. VRS·[^{14}C]Thr~AMP (1.7 μM, buffer as for Figure 1) was mixed with either 2.4 μM (●) or 22 μM (○) $tRNA^{Val}$. In a second experiment with 22 μM $tRNA^{Val}$, excess VRS was added after 2 min as indicated.

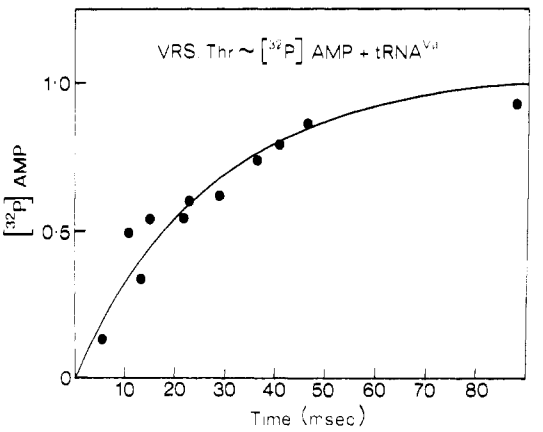

FIGURE 3: tRNA-stimulated decomposition of VRS·Thr~[^{32}P]AMP at 25 °C, pH 7.78, 10 mM $MgCl_2$, and 10 mM mercaptoethanol. Equal volumes of $tRNA^{Val}$ (34 μM) and VRS·Thr-[^{32}P]AMP (1.86 μM) were mixed in the quenched-flow apparatus and the rate of formation of [^{32}P]AMP was measured. The curve is calculated for a rate constant of 36 s^{-1}.

proaches that for the pyrophosphate exchange reaction under similar conditions (Table I).

Reactions of VRS·Thr~AMP with tRNA at 0 °C, pH 6.28. (a) Specificity for $tRNA^{Val}$. The addition of tRNA enriched in either $tRNA_1^{Val}$ or $tRNA_2^{Val}$ to the VRS·Thr~[^{32}P]AMP complex at 0 °C and pH 6.28 causes the complex to hydrolyze rapidly with a half-life of about 9 s (k = 0.075 s^{-1}). The hydrolysis is enzyme catalyzed, since Thr~AMP is far more stable in the absence of enzyme. The addition of tRNA enriched for $tRNA^{Phe}$ and $tRNA^{Tyr}$ does not stimulate the hydrolysis. Under similar conditions, about 76% of the [^{14}C]Val from the VRS·[^{14}C]Val~AMP complex is transferred to $tRNA^{Val}$. This behavior is very similar to that observed on the addition of $tRNA^{Ile}$ to the IRS·Val~[^{32}P]AMP complex (Baldwin and Berg, 1966).

(b) Transient Formation of [^{14}C]Thr-tRNA. Addition of fractions of tRNA enriched for $tRNA_1^{Val}$ or $tRNA_2^{Val}$, but not those containing $tRNA^{Phe}$ or $tRNA^{Tyr}$, to equimolar concentrations of VRS·[^{14}C]Thr~AMP gives a transient formation of [^{14}C]Thr-$tRNA^{Val}$, extrapolating back to a 5.7% transfer at zero time (Figure 1). Quenching the reaction mixture with phenol within a few seconds of mixing and extracting the tRNA gave a product that had been charged to 3.8% with [^{14}C]Thr.

Addition of excess enzyme (1.9 μM) to this partially misacylated $tRNA^{Val}$ (0.9 μM $tRNA^{Val}$, 0.034 μM [^{14}C]Thr-$tRNA^{Val}$) at 0 °C, pH 6.28, deacylated >99% of the tRNA in 7 s.

These results are consistent with Scheme I.

Scheme I

$$\text{VRS·Thr}\sim\text{AMP} \xrightarrow[0.075\ s^{-1}]{tRNA^{Val}} \text{VRS·Thr-}tRNA^{Val} + \text{AMP}$$

$$\text{VRS·Thr-}tRNA^{Val} \xrightarrow{\sim 1\ s^{-1}} \text{VRS} + \text{Thr} + tRNA^{Val}$$

(c) Competitive Inhibition of the Hydrolysis of [^{14}C]Thr-$tRNA^{Val}$ by $tRNA^{Val}$. Schreier and Schimmel (1972) showed that $tRNA^{Ile}$ competitively inhibits the IRS catalyzed deacylation of Ile-$tRNA^{Ile}$. A similar situation occurs here (Figure 2). On mixing 22 μM $tRNA^{Val}$ with 1.7 μM VRS·[^{14}C]Thr~AMP, some of the [^{14}C]Thr-tRNA that is formed dissociates from the enzyme and slowly hydrolyzes. On adding an excess of enzyme over $tRNA^{Val}$ so that competitive inhibition is no longer possible, the remaining [^{14}C]Thr-tRNA is completely hydrolyzed within 7 s (Figure 2).

Reactions of VRS·Thr~AMP with $tRNA^{Val}$ and the VRS-Catalyzed Deacylation of Thr-$tRNA^{Val}$ at 25 °C, pH 7.78. The rate constant for the hydrolysis of VRS·Thr~[^{32}P]AMP stimulated by $tRNA^{Val}$ is 36 s^{-1} (Figure 3). The rate constant for the VRS catalyzed hydrolysis of [^{14}C]Thr-tRNA is 40 s^{-1} (Figure 4). The addition of $tRNA^{Val}$ to VRS·[^{14}C]Thr~AMP gives a transient burst of [^{14}C] Thr-tRNA with a peak of 22% of the [^{14}C]Thr transferred at 25 ms, rapidly falling to zero after 150 ms (Figure 5). The time

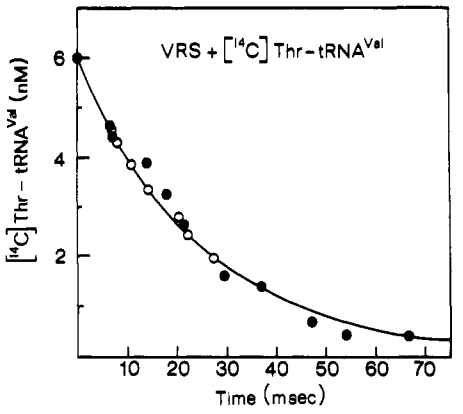

FIGURE 4: VRS-catalyzed hydrolysis of Thr-tRNA^{Val} at 25 °C, pH 7.78, 10 mM $MgCl_2$ and 10 mM mercaptoethanol. (●) Equal volumes of VRS (2 μM) and tRNA ([^{14}C]Thr-tRNA^{Val}, 12 nM; tRNA^{Val}, 320 nM) were mixed in the quenched flow apparatus. (○) Same as in ● but with ATP (4 mM), valine (800 μM), and inorganic pyrophosphatase (1 unit/ml) added to the enzyme to convert the VRS to VRS·Val~AMP. The curve is calculated for a rate constant of 40 s^{-1}.

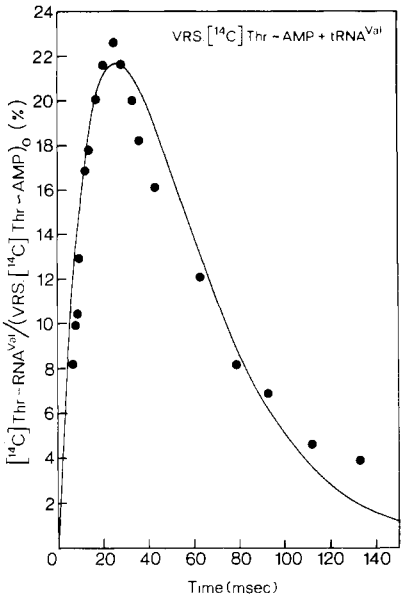

FIGURE 5: The transient formation of ["[^{14}C]Thr-tRNA^{Val} at 25 °C, pH 7.78, 10 mM $MgCl_2$, and 10 mM mercaptoethanol. Equal volumes of tRNA^{Val} (34 μM) and VRS·[^{14}C]Thr~AMP (1.8 μM) were mixed in the quenched-flow apparatus. The curve for the ratio of [^{14}C]Thr-RNA^{Val} to the initial concentration of VRS·[^{14}C]Thr~AMP is calculated for the kinetic scheme involving transfer of 62% of the [^{14}C]Thr from the complex to the tRNA at 36 s^{-1}, followed by the deacylation at 40 s^{-1}.

dependence of the concentration of [^{14}C]Thr-tRNA fits the theoretical curve constructed for Scheme II where 62% of the [^{14}C]Thr is transferred to the tRNA.

Scheme II

$$\text{VRS·[}^{14}\text{C]Thr} \sim \text{AMP·tRNA}^{Val} \xrightarrow{36\ s^{-1}} \text{VRS·[}^{14}\text{C]Thr-tRNA} \downarrow 40\ s^{-1} \quad \text{VRS} + [^{14}\text{C}]\text{Thr} + \text{tRNA}$$

These data may be compared with the rate constant of 12 s^{-1} with which 84% of the [^{14}C]Val from VRS·[^{14}C]-Val~AMP is transferred to tRNA (Figure 6), and 1.5×10^{-2} s^{-1} for the VRS-catalyzed hydrolysis of Val-tRNA^{Val}. The reason for these incomplete transfers is unknown (Baldwin and Berg, 1966).

The turnover number for the VRS-catalyzed hydrolysis of Thr-tRNA^{Val} is the highest yet reported (see Bonnet and Ebel, 1974).

The turnover number for the steady-state threonine stimulated ATP pyrophosphatase activity, 6 s^{-1}, is considerably less than the rate constant for the transfer step, 36 s^{-1}. This is possibly due to the formation of threonyl adenylate being partially rate determining, since the k_{cat} for the pyrophosphate exchange reaction is only 10 s^{-1}.

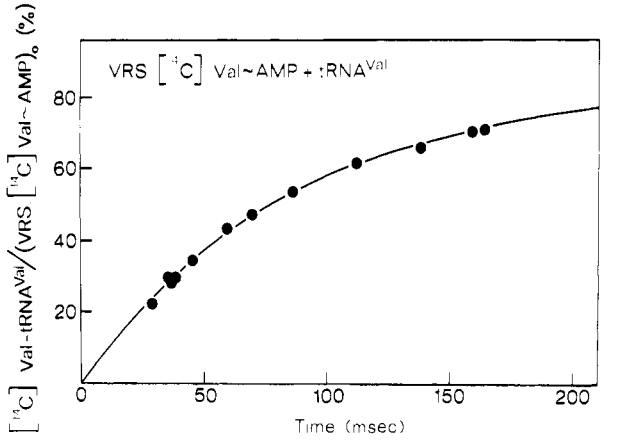

FIGURE 6: Transfer of [^{14}C]Val to tRNA^{Val} at 25 °C, pH 7.78, 10 mM $MgCl_2$, and 10 mM mercaptoethanol. Equal volumes of tRNA^{Val} (11 μM) and VRS·[^{14}C]Val~AMP (0.26 μM) were mixed in the quenched-flow apparatus. The curve is calculated for a rate constant of 11.7 s^{-1} and the transfer of 84% of the [^{14}C]Val.

Discussion

The addition of tRNA^{Val} to the VRS·[^{14}C]Thr~AMP complex leads to a transient production of [^{14}C]Thr-tRNA, reaching a maximum of 22% transfer at 25 ms (Figure 5). The time dependence of the concentration of the Thr-tRNA fits that predicted from the independently measured rate constants for the overall decomposition of the complex (36 s^{-1}) and the VRS-catalyzed hydrolysis of Thr-tRNA^{Val} (40 s^{-1}). The large fraction of misacylated tRNA formed, extrapolating to 62% of the total VRS·[^{14}C]Thr~AMP complex, rules out that this is due to impurities in the reagents. The enzyme prevents the net misacylation of tRNA by first mischarging the tRNA and then correcting the error with "editing" by hydrolysis.

Evidence Concerning the Mechanism. (a) There Are Two Separate Active Sites. The hydrolysis of the misacylated tRNA could be due to either a conformational change causing the acylation site to be converted to a hydrolytic site or the presence of a distinct second site. The latter appears to be the case, since we and others (Schreier and Schimmel, 1972; Yarus, 1972) find that the enzymes continue to hydrolyze aminoacylated tRNA, while the cognate aminoacyl adenylate occupies the acylation site. A second site would appear necessary, since otherwise, in vivo, any misacylated tRNA diffusing from the enzyme would be prevented from returning by the rapid formation of the enzyme bound aminoacyl adenylate.

(b) Deacylation Need Not be Preceded by Dissociation of the Enzyme-tRNA Complex. The following suggests that the deacylation occurs without the tRNA leaving the enzyme. Uncharged tRNA competitively inhibits the hydrolysis of the acylated tRNA (Figure 2). If the charged tRNA left the en-

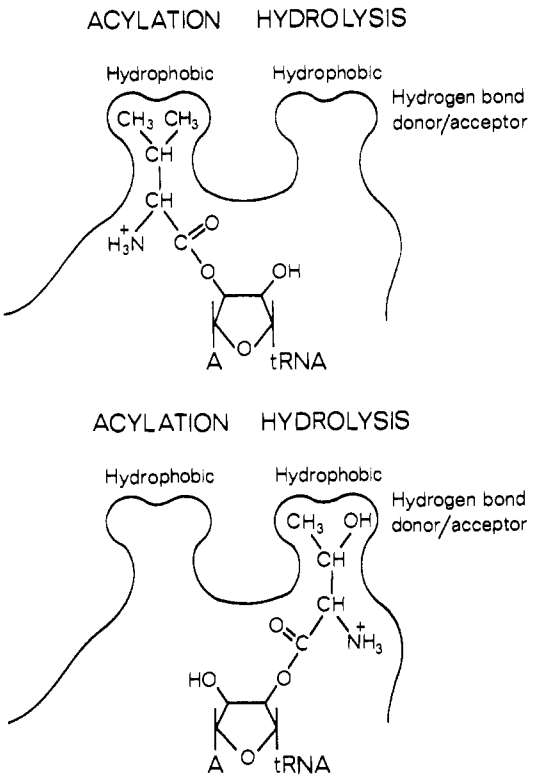

FIGURE 7: Illustration of the specificity mechanism. Top: the hydrophobic acylation site discriminates against threonine. Bottom: the hydrolytic site presumably specifically utilizes the hydroxyl of the threonine for a binding or catalytic effect. The translocation may occur via a 2′ → 3′-OH acyl transfer as illustrated or by a movement of the terminal adenosine.

zyme before hydrolyzing and then returned to a second site, then in the presence of excess uncharged tRNA a large fraction of charged tRNA would build up. However, as seen in Figure 2, only a small fraction of [^{14}C]Thr-tRNA^{Val} "leaks through" and hydrolyzes slowly due to the inhibition. Deacylation is faster than dissociation. (In vivo, since tRNA and the aminoacyl-tRNA synthetases are present in comparable amounts (Yarus and Berg, 1969), the competitive inhibition by uncharged or correctly charged tRNA should be of little consequence.)

The Translocation Step. The translocation of the aminoacyl ester to the hydrolytic site may be simply due to a movement of the CCA terminus of the tRNA. However, an alternative hypothesis may be devised from this study and a recent result from von der Haar and Cramer (1975). They find that on replacing the terminal adenosine of yeast tRNA^{Ile} with 3′-deoxyadenosine, the modified tRNA is still charged by the yeast IRS and ATP. However, it will now also accept valine. Since it is known that aminoacyl groups are rapidly and reversibly transferred between the 2′- and 3′-hydroxyl groups of the ribose ($t_{1/2} \sim 0.2$ ms, Griffin et al., 1966), it is possible that for the IRS and the VRS the acylation site is on the 2′-OH side of the ribose, while the hydrolytic site is on the 3′-OH side so that translocation simply involves the intramolecular migration of the aminoacyl moiety (Figure 7).

Hyperspecificity Depends on Using Structural Differences Twice. Whatever the detailed mechanism of the reaction, the discrimination between valine and threonine depends on the relative rates of acylation and hydrolysis. The enzyme attains its high specificity by using the differences in the structure of the competing substrates *twice:* once by discriminating against the undesired uubstrate in the synthetic step, and once by discriminating against the desired substrate in the destructive step.

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