Hydrolytic Action of Aminoacyl-tRNA Synthetases from Baker's Yeast. "Chemical Proofreading" of Thr-tRNA^{Val} by Valyl-tRNA Synthetase Studied with Modified tRNA^{Val} and Amino Acid Analogues†

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ABSTRACT: The properties of native and of two modified tRNA^{Val} species in the correction of misactivated threonine by valyl-tRNA synthetase have been studied. Whereas Thr-tRNA^{Val}-C-C-A could not be isolated in the valyl-tRNA synthetase catalyzed reaction, Thr-tRNA^{Val}-C-C-3′dA is isolable in up to 50% yield in this system and tRNA^{Val}-C-C-3′NH$_2$A is fully aminoacylated with threonine by the same enzyme. The hydrolysis of preformed Thr-tRNA^{Val}-C-C-A by free valyl-tRNA synthetase is 30 times faster than the corresponding breakdown of Val-tRNA^{Val}-C-C-A. This hydrolytic activity is also observed with Thr-tRNA^{Val}-C-C-3′dA although the rate is reduced to that of the reaction of Val-tRNA^{Val}-C-C-A. Modification of the threonine to *O*-methylthreonine, which is also a substrate for valyl-tRNA synthetase, leads to stabilization of the *O*-methylthreonyl-tRNA esters. The AMP/PP independent hydrolysis under aminoacylating conditions, which is a measure of the correction process, indicates that *O*-MeThr-tRNA^{Val}-C-C-A is only very slowly corrected while the tRNA^{Val}-C-C-3′dA and tRNA^{Val}-C-C-3′NH$_2$A esters are completely stable. Removal of the methoxy group of *O*-methylthreonine as in α-aminobutyric acid increases the rate of the hydrolytic reaction and once again α-Abu-tRNA^{Val}-C-C-A and α-Abu-tRNA^{Val}-C-C-3′dA are unstable under aminoacylating conditions and not isolable.

A major aspect of aminoacyl-tRNA synthetase action in protein biosynthesis is the positioning of one particular amino acid on the correct tRNA. Mischarging at this stage in the pathway leads inevitably to conservation of the error and incorporation of the wrong amino acid into the protein, since during the remaining steps of protein biosynthesis there is no further check on the fidelity of aminoacylation. The specificity of the enzymes is usually maintained at the level of activation (eq 1), and only the cognate amino acid will be transformed into the aminoacyl adenylate intermediate. Several enzymes, however, are exceptions to this general observation. Thus of the best known examples, isoleucyl-tRNA synthetase will activate valine and valyl-tRNA synthetase activates threonine, although neither incorrectly charged tRNA is formed (Baldwin and Berg, 1966; Fersht and Kaethner, 1976). For this reason an enzymatic correction step during tRNA aminoacylation (eq 2) has been envisaged (Eldred and Schimmel, 1972; Hopfield et al., 1976).

$$\text{aa} + \text{ATP} + \text{E} \rightleftharpoons [\text{E} \cdot \text{aa-AMP}] + \text{PP} \quad (1)$$

$$[\text{E} \cdot \text{aa-AMP}] + \text{tRNA} \rightleftharpoons \text{aa-tRNA} + \text{AMP} + \text{E} \quad (2)$$

The misactivation of valine by isoleucyl-tRNA synthetase has been studied in depth (von der Haar and Cramer, 1975) and a mechanism of chemical proofreading has been proposed (von der Haar and Cramer, 1976). During this process a water molecule, which occupies the area on the enzyme where the methyl group of isoleucine is usually located, is enzymically activated via the 3′-OH of the terminal adenosine of tRNA^{Ile} followed by nucleophilic attack on the biologically incorrect ester bond. In the case of valyl-tRNA synthetase catalyzed misactivation of threonine the structural difference between valine and threonine is the replacement of CH_3 by OH. Thus, the above mechanism involving the insertion of a water molecule into a vacated area may not be applicable and the question arises of whether a completely different correction mechanism takes place.

We have been investigating this system and report here the effect of modified substrates on the aminoacylation of tRNA^{Val} with threonine by valyl-tRNA synthetase. We interpret our findings in terms of a second example of chemical proofreading.

Experimental Section

Materials

The purification of valyl-, phenylalanyl-, and isoleucyl-tRNA synthetases (EC 6.1.1.9, 6.1.1.20, 6.1.1.5) as well as the isolation of tRNA^{Val} and its 3′-terminal modification have been described (von der Haar and Cramer, 1976). Threonyl-tRNA synthetase (EC 6.1.1.3) from baker's yeast was purified in this laboratory to apparent homogeneity (the specific activity of 463 units/A_{280} unit was determined below the pH optimum of approximately 8.5 at pH 7.5) by a series of column chromatographic steps including the use of fractional interfacial salting out (von der Haar, 1976a). [^{32}P]Pyrophosphate and [^{14}C]ATP (specific activity 81 and 47 mCi/mmol, respectively) were purchased from the Radiochemical Centre (Amersham, England). ^{14}C-Labeled amino acids of Stanstar grade (50 mCi/mmol) were a product of Schwarz Bio-Research, Inc. (Orangeburg, N.Y.). L-*O*-Methylthreonine was from Calbiochem (San Diego, Calif.) and L-α-aminobutyric acid was obtained from Fluka AG. All other reagents were commercial analytical grade products.

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ATP/PP Exchange

The assay using [^{32}P]pyrophosphate was performed as described (Simlot and Pfaender, 1973; von der Haar and Gaertner, 1975).

Aminoacylation of tRNA

The standard charging of tRNA in homologous systems was carried out by the method of von der Haar and Cramer (1976). In the case of mischarging of native or modified tRNA^{Val} the conditions of the reaction were optimized with respect to the charging efficiency.

(a) *Preparative Charging of tRNA^{Val}-C-C-3′dA*[1] *by Valyl-tRNA Synthetase with Threonine.* The incubation, in a total of 1 mL, contained 150 mM Tris-HCl (pH 7.6), 50 mM KCl, 10 mM $MgSO_4$, 1.5 mM ATP, 60 μM [^{14}C]threonine, 0.18 mg of valyl-tRNA synthetase, and 7.5 μM tRNA^{Val}-C-C-3′dA. The reaction was maintained at 37 °C for 10 min and the tRNA was isolated as described (von der Haar and Cramer, 1976).

(b) *Preparative Charging of tRNA^{Val}-C-C-3′NH$_2$A by Valyl-tRNA Synthetase with Threonine.* The incubation in a total of 1 mL contained 150 mM Tris-HCl (pH 7.6), 50 mM KCl, 10 mM $MgSO_4$, 20 μM [^{14}C]threonine, 2 mM ATP, 78 μg of valyl-tRNA synthetase, and 7.5 μM tRNA^{Val}-C-C-3′NH$_2$A. The reaction was continued for 60 min at 37 °C and the product isolated as above.

(c) *Preparative Charging of tRNA^{Val}-C-C-A by Threonyl-tRNA Synthetase and Threonine.* Incubation in 1 mL contained 10 mM Tris-HCl (pH 9.0), 8 mM $MgSO_4$, 0.5 mM ATP, 60 μM [^{14}C]threonine, 4.5 μM tRNA^{Val}-C-C-A, 20% methanol, and 80 μg of threonyl-tRNA synthetase from baker's yeast. Incubation was for 20 min at 37 °C.

(d) *Charging of tRNA^{Val}-C-C-N with Unlabeled Amino Acids and Estimation of the Residual Free tRNA by Back-Titration with [^{14}C]Valine.* In the case of, for example, *O*-methylthreonine where no radioactively labeled compound is commercially available direct measurement of charging of tRNA is not possible without an analysis of the resulting tRNA molecule. For this reason the principle of back-titration was used to determine the amount of uncharged tRNA remaining after aminoacylation of tRNA with certain unlabeled amino acids.

Unless otherwise stated 1.2–1.5 nmol of tRNA^{Val}-C-C-N was preincubated in a total volume of 100 μL with 150 mM Tris-HCl (pH 7.6), 50 mM KCl, 10 mM $MgSO_4$, 2 mM ATP, and 0.2 mM unlabeled amino acid in the presence of 7.8 μg of valyl-tRNA synthetase for varying lengths of time at 37 °C. At the end of the specified period the residual uncharged tRNA was assayed by the addition of 10 μL of a solution containing 0.5 mM [^{14}C]valine and 5 mM ATP. A 10-μL aliquot was immediately withdrawn to give a T = 30 s point in the [^{14}C]valine charging reaction. A further 7.8 μg of valyl-tRNA synthetase (in 1 μL) was then added and the incubation continued at 37 °C, assaying [^{14}C]valine incorporation at given time intervals by the method described (Schlimme et al., 1969).

AMP/PP independent hydrolyses of aminoacyl-tRNAs both with free enzyme and under aminoacylating conditions were followed as described previously (von der Haar and Cramer, 1976). The calculation of the turnover numbers from these data was also defined in the same publication.

Estimation of the Amount of Threonine in the Commercial O-Methylthreonine

The method of Neidig and Hess (1952) was adopted. Oxidation of threonine took place in a solution containing the sample in 5 mL of water, 15 mL of 0.05 M sodium borate buffer (pH 8), and 1 mL of 5% sodium periodate. The acetaldehyde formed during the reaction was aerated with dry air, with the help of a filter pump, into the color reagent solution containing 1 mL of water, 6 mL of H_2SO_4, 1 drop of 4% $CuSO_4$, and 0.2 mL of 1.5% *p*-hydroxybiphenyl in 0.5% NaOH. This trap was kept in ice during the 60 min of oxidation. After this time the trap was warmed at 80–90 °C for a few minutes to dissolve the excess biphenyl reagent. The resulting purple/blue color was measured at the λ_{max} of 575 nm. The absorbance obtained in this way from the *O*-methylthreonine sample was compared with the oxidation product from threonine of known concentration. The absolute concentrations of the amino acids were determined by the fluorimetric method of Roth (1971), using tryptophan as an optical standard.

Preparative Oxidation of Threonine Contamination in the O-Methylthreonine

O-Methylthreonine (10 mg) in 5 mL of water was incubated with 15 mL of borate buffer described above and 1 mL of 5% sodium periodate in the dark for 60 min at room temperature. After this time the excess periodate was destroyed by the additon of glucose and the solution evaporated to dryness. The solid residue was redissolved in water and ninhydrin positive material was isolated using a Dowex 50W-X2 column. The amino acid was desalted by extraction of the dried eluate with ethanol. The ethanol was evaporated and the *O*-methylthreonine dissolved in water. The product ran as a single spot on cellulose coated thin-layer sheets (Macherey and Nagel, Düren, Germany) in a solvent of BuOH–AcOH–H_2O (4:1:1) to the same position as the major spot from the commercial *O*-methylthreonine.

Results

Effect of tRNA Modification on Valyl-tRNA Synthetase Catalyzed Aminoacylation with Threonine. A series of experiments was carried out to determine how modification of the 3′-C-C-A end of tRNA^{Val} affected its misacylation with threonine by valyl-tRNA synthetase. In the homologous aminoacylation system Thr-tRNA^{Val}-C-C-A could not be isolated. Of the modified tRNAs, tRNA^{Val}-C-C-3′NH$_2$A was fully and stably charged with threonine (Table I, Figure 1) but Thr-tRNA^{Val}-C-C-3′dA could only be isolated in a 20–30% yield depending on enzyme and tRNA concentrations (Table I). The intermediate position of tRNA^{Val}-C-C-3′dA in this series of reactions was a first indication that in this case a competition between charging and deacylation (as discussed further below) could be occurring. Table I summarizes these findings and Figure 1 shows how the stability of these charged, isolated tRNA species is decreased by free valyl-tRNA synthetase during AMP/PP independent hydrolysis.

Since, as also found in the isoleucyl-tRNA synthetase/valine system (Schreier and Schimmel, 1972), uncharged tRNA^{Val} is a potent inhibitor of the AMP/PP independent hydrolysis (50% inhibition is at a fourfold excess of uncharged tRNA^{Val}), to be able to compare the initial rates of hydrolysis for the

[1] Abbreviations used are: A, adenosine; 2′dA, 2′-deoxyadenosine; 3′dA, 3′-deoxyadenosine; F, formycin; 3′NH$_2$A, 3′-deoxy-3′-aminoadenosine; Xxx-tRNA^{Yyy}-C-C-N, tRNA specific for the amino acid Yyy fitted with the terminal nucleoside N and aminoacylated with Xxx; *O*-MeThr, *O*-methylthreonine; *O*-MeThr$_{oxi}$, periodate-oxidized *O*-methylthreonine to destroy contaminating threonine; α-Abu, L-α-aminobutyric acid.

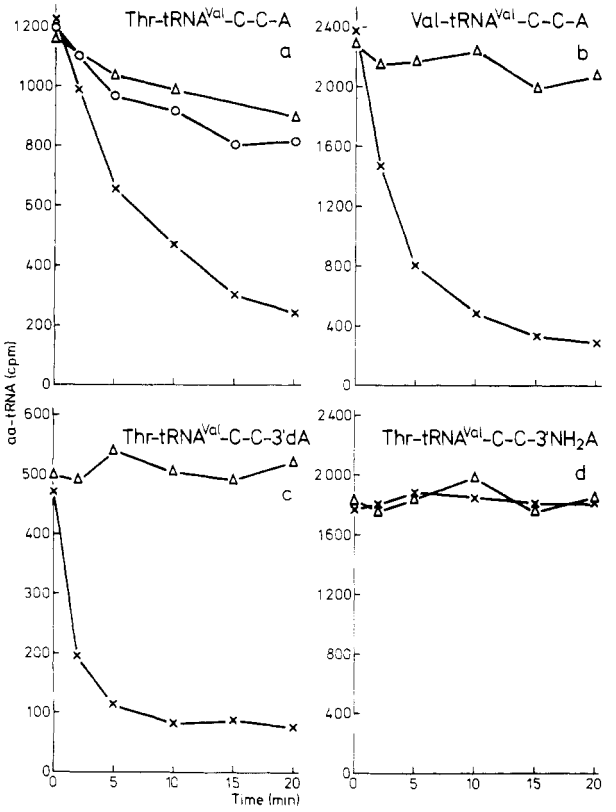

FIGURE 1: AMP/PP independent hydrolysis of aminoacyl-tRNA^{Val}-C-C-N by free valyl-tRNA synthetase. Results were obtained as described in the Experimental Section. In each case △ represents spontaneous hydrolysis of aminoacyl-tRNA in the absence of enzyme. In enzyme-induced hydrolysis the concentration of valyl-tRNA synthetase (×) was 3 nM (a) and 0.3 μM (b, c, d) and the concentration of phenylalanyl-tRNA synthetase (○) was 0.2 μM.

TABLE I: Aminoacylation of tRNA^{Val}-C-C-N by Valyl- and Threonyl-tRNA Synthetase with Threonine under Conditions Described in the Experimental Section.

	nmol of Threonine/A_{260} Unit of tRNA	
tRNA^{Val}-C-C-N N =	Valyl-tRNA Synthetase	Threonyl-tRNA Synthetase
A	0.013	0.5-0.8
F	0.052	0.02
2'dA	<0.01	0.03
3'dA	0.3-0.5	0.04
3'NH$_2$A	1.6	<0.01

various aminoacyl-tRNAs correction has to be made for the nonacylated tRNA present in the reaction. The effect of tRNA^{Val}-C-C-A on the AMP/PP independent hydrolysis of Val-tRNA^{Val}-C-C-A by valyl-tRNA synthetase was determined (Figure 2). By assuming that uncharged tRNA^{Val}-C-C-N has a similar effect on the hydrolysis of aminoacyl-tRNA^{Val}-C-C-N a correction factor could be applied. The turnover numbers quoted in Table II are corrected for both this inhibitory effect on the initial rates of hydrolyses and also for spontaneous breakdown of the aminoacyl-tRNAs.

Comparison with the corresponding Val-tRNA^{Val}-C-C-N compounds (Figure 1, Table III) shows that, as was found for the isoleucyl-tRNA synthetase/valine system (von der Haar and Cramer, 1976), Thr-tRNA^{Val}-C-C-A is hydrolyzed much

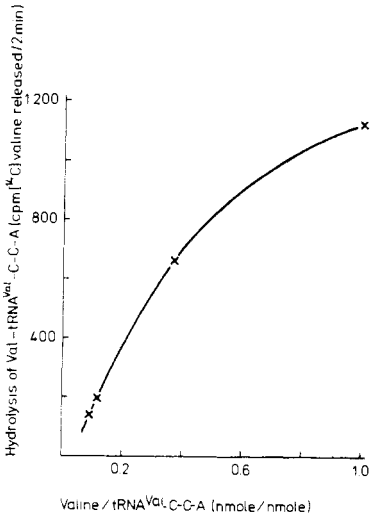

FIGURE 2: Inhibition of AMP/PP independent hydrolysis of Val-tRNA^{Val}-C-C-A by tRNA^{Val}-C-C-A. Data were obtained as described in the Experimental Section. Enzyme concentration was 0.3 μM and 25-μL aliquots were removed from the incubation for counting.

more rapidly than Val-tRNA^{Val}-C-C-A. Furthermore, this AMP/PP independent hydrolysis is a specific property of valyl-tRNA synthetase as shown by the stability of the mischarged species in the presence of phenylalanyl- and isoleucyl-tRNA synthetases. The tRNA^{Val}-C-C-3'dA derivative which is stable when charged with valine (von der Haar and Cramer, 1976) is, when aminoacylated with threonine, hydrolyzed by valyl-tRNA synthetase but at a slower rate than Thr-tRNA^{Val}-C-C-A. This, moreover, confirmed the idea proposed above that during aminoacylation of tRNA^{Val}-C-C-3'dA one is observing the result of competition between charging and breakdown of the tRNA ester. Thus, there is an aminoacylation of the 2'-OH of the terminal tRNA ribose and this is followed by the enzymatic splitting of the amino acid from the tRNA as shown in Figure 1c. The nonstoichiometric yield of isolable Thr-tRNA^{Val}-C-C-3'dA (Table I) corresponds to the equilibrium set up between these reactions, and the sensitivity of this equilibrium to, for example, tRNA concentrations is indicated by the variation of the amount of the product obtained under different conditions. Both Val- and Thr-tRNA^{Val}-C-C-3'NH$_2$A are stable in this reaction (Figure 1d).

To examine the forward reaction of aminoacylation under dynamic conditions, the effect of the above-modified tRNAs on ATP hydrolysis during aminoacylation by threonine was investigated. As described in detail previously (von der Haar and Cramer, 1976), the AMP/PP independent hydrolysis of aminoacyl-tRNA also occurs under aminoacylation conditions giving rise to a continuous hydrolysis of ATP as the liberated tRNA is reaminoacylated in a cyclic process. The formation of AMP during the charging reaction in amounts far in excess of the stoichiometry required for one round of aminoacylation is evidence that such a charging/hydrolysis process is occurring. Figure 3 and Table III show the results obtained during the course of these experiments. The threonine-dependent hydrolysis which takes place under aminoacylating conditions is much faster than the corresponding reaction supported by valine. From the threonine concentration dependence of the process a K_m for threonine in the overall reaction of 20 μM is found which is close to the K_m for valine in aminoacylation and is a markedly reduced value from the K_m for threonine in pyrophosphate exchange (Table IV).

TABLE II: Corrected Turnover Numbers for AMP/PP Independent Hydrolysis of aa-tRNA^{Val}-C-C-N by Free Aminoacyl-tRNA Synthetases.[a]

Aminoacyl-tRNA Synthetase	aa-tRNA^{Val}-C-C-N	Spontaneous Hydrolysis (nmol min^{-1} L^{-1})	Turnover No. (min^{-1})
Valyl-	Val-tRNA^{Val}-C-C-A	5	1.2 (0.77)[b]
Valyl-	Val-tRNA^{Val}-C-C-3′dA	(<1)[b]	(0.07)[b]
Valyl-	Thr-tRNA^{Val}-C-C-A	9	32.0
Valyl-	Thr-tRNA^{Val}-C-C-3′dA	<1	2.4
Valyl-	Thr-tRNA^{Val}-C-C-3′NH$_2$A	<1	<0.01
Phenylalanyl-	Thr-tRNA^{Val}-C-C-A	9	0.1
Isoleucyl-[c]	Thr-tRNA^{Val}-C-C-A	9	0.4

[a] 150 mM Tris-HCl (pH 7.6), 150 mM KCl, 10 mM $MgSO_4$, 2.5 μM [^{14}C]aa-tRNA; enzyme concentration as described in Figure 1; incubation at 37 °C in total volume of 200 μL. [b] From von der Haar and Cramer (1976). [c] Omitted from Figure 1 for the sake of clarity.

TABLE III: Turnover Numbers for AMP/PP Independent Hydrolysis of aa-tRNA by Valyl-tRNA Synthetase under Aminoacylation Conditions.[a]

tRNA^{Val}-C-C-N	[tRNA] (μM)	Amino Acid	Turnover No. (min^{-1})
tRNA^{Val}-C-C-A	5.4	Val	0.85 (1.0)[b]
	5.4	Thr	5.2
	4.8	*O*-MeThr$_{oxi}$	0.28
	4.7	α-Abu	11.0
tRNA^{Val}-C-C-3′dA	4.8	Val	(0.08[b])
	4.8	Thr	2.15
	8.1	*O*-MeThr	<0.01
	4.7	α-Abu	3.3
tRNA^{Val}-C-C-3′NH$_2$A	13.2	Val	<0.01[c]
	13.2	Thr	0.3[c]
	13.2	*O*-MeThr	<0.01[c]
	4.3	α-Abu	<0.05[c]

[a] 150 mM Tris-HCl (pH 7.6), 150 mM KCl, 10 mM $MgSO_4$, 1 mM amino acid, 0.5 mM [^{14}C]ATP; enzyme concentration as described in Figures 3 and 4; incubation at 37 °C. [b] From von der Haar and Cramer (1976). [c] Values for AMP formed due to stoichiometric tRNA charging were subtracted from the observed initial rates.

TABLE IV: Michaelis Constants for Amino Acid Substrates of Valyl-tRNA Synthetase in ATP/PP Exchange.

Amino Acid	K_m (mM)	Rel V_{max} (%)
Val	0.17	100
Thr	3.1	42
O-MeThr	10.0	18
α-Abu	2.8	20

In contrast to Val-tRNA^{Val}-C-C-3′dA, there is a relatively rapid removal of threonine from Thr-tRNA^{Val}-C-C-3′dA (as measured by AMP formation, Figure 3) and this can again be correlated with the similar lability of the Thr-tRNA^{Val}-C-C-3′dA compound to free enzyme (see above).

The slow but significant ATP hydrolysis during aminoacylation of tRNA^{Val}-C-C-3′NH$_2$A with threonine (Figure 3f) is of interest. It means that although tRNA^{Val}-C-C-3′NH$_2$A is charged with threonine (Table I) some deacylation must occur from the 2′-acceptor OH prior to transacylation of the amino acid onto the 3′NH$_2$. In this case and in contrast to the reaction involving tRNA^{Val}-C-C-3′dA described above, a third

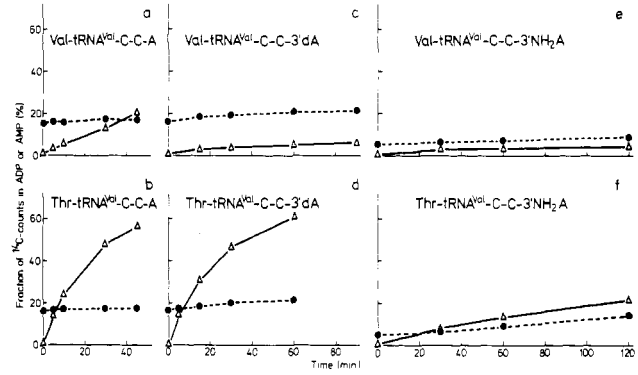

FIGURE 3: AMP/PP independent hydrolysis of Val- and Thr-tRNA^{Val}-C-C-N by valyl-tRNA synthetase under aminoacylation conditions. Details of the procedure are described in von der Haar and Cramer (1976). The tRNA concentrations are shown in Table III. In each case AMP formation is indicated by Δ-Δ-Δ and ADP, which is present in all commercial samples of [^{14}C]ATP to an extent of 5–15%, is monitored by ● - - - ●. Valyl-tRNA synthetase concentrations were 2.4 μM (a, b, e, f) and 6.1 μM (c, d).

reaction pathway, transacylation to form the stable 3′-amide (Figure 1), occurs and after a number of enzyme cycles the buildup of this product will be the overall observed reaction. The Val-tRNA^{Val}-C-C-3′NH$_2$A does not take part in such a recycling process (Figure 3e).

Effect of tRNA Modification on Valyl-tRNA Synthetase Catalyzed Aminoacylation with Threonine Analogues. *O*-Methylthreonine, where the side-chain OH of threonine is methylated, is a substrate in the pyrophosphate exchange reaction catalyzed by valyl-tRNA synthetase. The K_m (Table IV) is high and the V_{max} is reduced compared with both valine and threonine. Nevertheless, it was of interest to examine what effect, if any, the blocking of the free OH group had on the pattern of the aminoacylation/deacylation process described above. Since *O*-methylthreonine is not available commercially in a radioactive form, the first approach in evaluating its potential in aminoacylation was to examine the AMP/PP independent hydrolysis under aminoacylation conditions. Figure 4a–d shows the nature of the results. There is no rapid AMP formation comparable with the threonine-dependent reactions with any of the three tRNAs of interest. In the case of tRNA^{Val}-C-C-A the slow net ATP hydrolysis observed (Figure 4a) is not due to the threonine contaminant present in the commercial *O*-methylthreonine sample. Although a contamination of 1.6% threonine was found (see Experimental Section) and this amount of threonine could be expected to con-

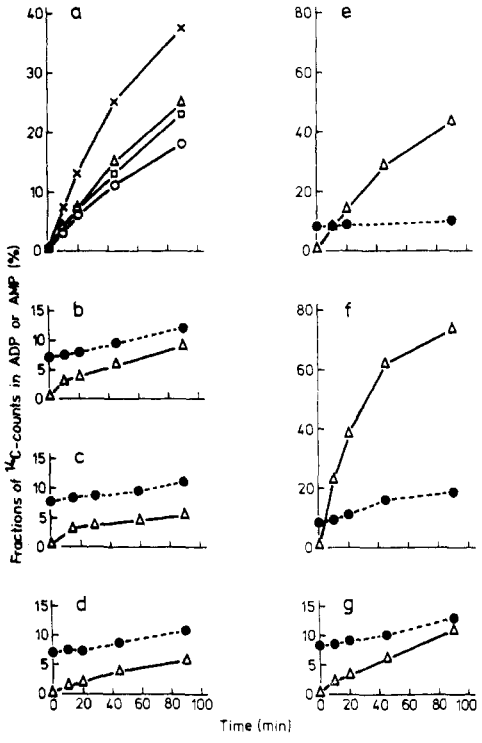

FIGURE 4: AMP/PP independent hydrolysis of *O*-MeThr-tRNA^{Val}-C-C-N and α-Abu-tRNA^{Val}-C-C-N by valyl-tRNA synthetase under aminoacylating conditions. Incubations were as described in the Experimental Section: changes in AMP levels (△-△); changes in ADP levels (● - - - ●). The enzyme concentration was 6.5 μM except in e (0.65 μM). (a) *O*-Methylthreonine and 4.8 μM tRNA^{Val}-C-C-A; additionally compared with AMP formation curves at 10 μM threonine (○-○), 50 μM threonine (×-×), and 1 mM oxidized *O*-methylthreonine (□-□); the ADP curve which was identical with those in b, c, and d has been omitted for clarity; (b) *O*-methylthreonine and 8.1 μM tRNA-^{Val}-C-C-3′dA; (c) *O*-methylthreonine and 2.4 μM tRNA^{Val}-C-C-3′NH$_2$A; (d) no tRNA; (e) α-aminobutyric acid and 4.7 μM tRNA^{Val}-C-C-A; (f) α-aminobutyric acid and 4.7 μM tRNA^{Val}-C-C-3′dA; (g) α-aminobutyric acid and 4.3 μM tRNA^{Val}-C-C-3′NH$_2$A.

tribute to the ATP splitting observed (Figure 4a), periodate-oxidized *O*-methylthreonine, in which the level of threonine was reduced to 0.25% without affecting the *O*-methylthreonine, still supported the AMP/PP independent hydrolysis under the conditions described in Figure 4a. It seems, therefore, that in only one of the cases tested does *O*-methylthreonine take part in an aminoacylation/deacylation cycle.

An alternative to blocking of the OH functional group is its complete removal. Thus, L-α-aminobutyric acid, where the threonine hydroxyl is replaced by H, is a substrate for valyl-tRNA synthetase catalyzed ATP-PP exchange. The kinetic constants for this reaction (Table IV) are similar to those of the *Escherichia coli* enzyme (Bergmann et al., 1961). The activity of α-aminobutyric acid in AMP/PP independent hydrolysis under aminoacylation conditions in the presence of tRNA^{Val}-C-C-A (Figure 4e) is in agreement with the finding of Freundlich (1967) in the *E. coli* system. That is, the rapid AMP formation corresponds to the aminoacylation/correction cycle and with the inability of Freundlich (1967) to observe a stably charged α-Abu-tRNA^{Val}-C-C-A. We also find a similar correction process when tRNA^{Val}-C-C-3′dA is the accepting species (Figure 4f), although the rate of ATP splitting is reduced to a third (Table III). tRNA^{Val}-C-C-3′NH$_2$A probably does not take part in the AMP/PP independent hydrolysis (Figure 4g).

To determine whether the lack of activity of these modified

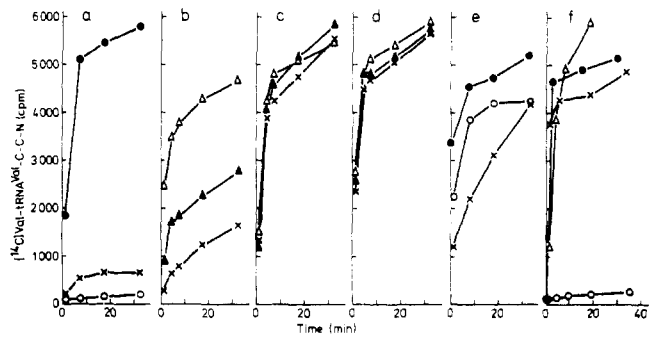

FIGURE 5: Back-titration with [^{14}C]valine/valyl-tRNA synthetase of residual free tRNA^{Val}-C-C-N after preincubation with unlabeled amino acids under aminoacylating conditions. Unless otherwise stated, conditions were as described in the Experimental Section: (a) preincubation with 11 μM tRNA^{Val}-C-C-3′NH$_2$A and no amino acid (●), *O*-methylthreonine (×), or threonine (○) for 60 min; (b) preincubation with 7.6 μM tRNA^{Val}-C-C-3′dA and *O*-methylthreonine for 10 min (△), 30 min (▲), and 60 min (×); (c) preincubation with 7.6 μM tRNA^{Val}-C-C-3′dA and threonine for 10 min (△), 30 min (▲), and 60 min (×); (d) preincubation with 7.6 μM tRNA^{Val}-C-C-3′dA and either no amino acid or no enzyme, for 10 min (△), 30 min (▲), and 60 min (×); (e) preincubation with 12 μM tRNA^{Val}-C-C-A and no amino acid (●), threonine (○), or *O*-methylthreonine (×) for 60 min; (f) preincubation for 30 min with 4.7 μM tRNA^{Val}-C-C-A and no amino acid (●) or 0.1 mM α-aminobutyric acid (×); preincubation for 30 min with 3.9 μM tRNA^{Val}-C-C-3′dA and 0.1 mM α-aminobutyric acid (△); preincubation for 30 min with 4.4 μM tRNA^{Val}-C-C-3′NH$_2$A and 0.1 mM α-aminobutyric acid (○); back-titration in f was with 80 μM [^{14}C]valine.

substrates in the AMP/PP independent hydrolysis was merely due to an intrinsic difficulty in forming an *O*-MeThr-tRNA^{Val}-C-C-N or α-Abu-tRNA^{Val}-C-C-N intermediate a method was devised by which the presence and extent of these misacylated species could be detected. The method is based on the principle of back-titration, in that the extent of the reaction at any given time is measured by the amount of the residual unchanged reactant. In this way tRNA^{Val}-C-C-N could be incubated under aminoacylating conditions with *O*-methylthreonine and, after a given time, any uncharged tRNA would be estimated by the addition of [^{14}C]valine. The formation of [^{14}C]Val-tRNA^{Val}-C-C-N (determined in the usual way (Schlimme et al., 1969)) was a direct measure of the amount of free tRNA^{Val}-C-C-N remaining and therefore, by difference, an estimate of the amount of unlabeled aminoacyl-tRNA^{Val}-C-C-N.

The amount of nonradioactive amino acid bound at the instant of adding [^{14}C]valine can be determined by extrapolation. A plateau in the amount of [^{14}C]Val-tRNA^{Val}-C-C-N is not always observed but after the initial rapid charging process a slow time-dependent increase in the amount of [^{14}C]Val-tRNA^{Val}-C-C-N is obtained (Figure 5). At least two processes not related to correction can be envisaged which could lead to this. Firstly, spontaneous hydrolysis of the ester bond between the amino acid and tRNA occurs and the free tRNA would be rapidly reaminoacylated with its cognate amino acid. The second process which might contribute to this effect is due to the accumulation of AMP as any small amount of Val-tRNA^{Val}-C-C-A or Val-tRNA^{Val}-C-C-3′dA goes through its acylation/hydrolysis cycle. The AMP would then be expected to induce a reversal of aminoacylation and the unlabeled amino acid would be lost from the tRNA. Because of these nonspecific hydrolytic mechanisms, an estimate of the amount of tRNA charged during the preincubation must be obtained by extrapolation of the back-titration curve to time $t = 0$.

Figures 5a-e show the results of such back-titrations during

the valyl-tRNA synthetase catalyzed aminoacylation of $tRNA^{Val}$-C-C-N with *O*-methylthreonine or threonine. The case of $tRNA^{Val}$-C-C-3′NH_2A (Figure 5a) is, perhaps, the easiest to interpret since here there is no loss of unlabeled amino acid from tRNA due to hydrolysis. It is clear that this modified tRNA does become charged with *O*-methylthreonine and to an extent of 91%. This compares with a yield of 97% for aminoacylation with threonine in the same system. The control incubation in the absence of either amino acid or valyl-tRNA synthetase gives maximal [^{14}C]valine incorporation when the missing component is added.

Comparison with the control curves (Figure 5d) shows that preincubation with threonine gives only little if any stably bound Thr-$tRNA^{Val}$-C-C-3′dA (Figure 5c). *O*-Methylthreonine, on the other hand, gives a product which is both resistent to hydrolysis and also increases in amount as the preincubation time is lengthened (Figure 5b). After 60-min preincubation 83% $tRNA^{Val}$-C-C-3′dA has been converted to *O*-MeThr-$tRNA^{Val}$-C-C-3′dA.

O-MeThr-$tRNA^{Val}$-C-C-A appears to be the most easily hydrolyzed of the *O*-methylthreonyl esters tested in this way (Figure 5e), but there is an undoubtable buildup of this product if one compares it with the behavior of threonine in the identical reaction.

The curves in Figure 5f confirm that neither $tRNA^{Val}$-C-C-A nor $tRNA^{Val}$-C-C-3′dA is stably charged with α-aminobutyric acid. $tRNA^{Val}$-C-C-3′NH_2A, on the other hand, is completely aminoacylated with this amino acid, as expected from the AMP/PP independent hydrolysis data (Figure 4g).

Discussion

As described in the introductory statement and discussed elsewhere (von der Haar and Cramer, 1975) fidelity of aminoacyl-tRNA synthetases is maintained in the case of isoleucyl- and valyl-tRNA synthetase by a postactivation correction process. Mechanism for both kinetic (Hopfield et al., 1976) and chemical proofreading (von der Haar and Cramer, 1976) have been put forward and while some generalizations have been made with regard to a kinetic process, both types of investigation have, so far, concerned solely the isoleucyl-tRNA synthetase/valine system (von der Haar, 1976b). To gain more information about the postactivation corrective activity of these enzymes the case of misactivation of threonine by valyl-tRNA synthetase has been studied. The use of modified tRNAs which have also proved to be a valuable tool in other areas (see, e.g., Cramer et al., 1975; von der Haar and Cramer, 1975) was the basis for these investigations.

Valine and threonine esters of $tRNA^{Val}$-C-C-N, where N = A, 3′dA, or 3′NH_2A, were investigated. A comparison of both the extent of charging and the rate of AMP/PP independent hydrolysis of these aminoacyl-$tRNA^{Val}$-C-C-N by free valyl-tRNA synthetase reveals a number of significant differences. $tRNA^{Val}$-C-C-N is charged in all three cases to 100% capacity with valine but only when N = A is there a measurable AMP/PP independent hydrolysis of aminoacylated tRNA by free valyl-tRNA synthetase. When threonine replaces valine in these charging reactions N = 3′NH_2A is aminoacylated to 100% and N = 3′dA to about 20–30% depending on the conditions, whereas with the native N = A esterification is not observed. Thr-$tRNA^{Val}$-C-C-3′dA, in contrast to the valine ester (von der Haar and Cramer, 1976), is sensitive to AMP/PP independent hydrolysis by free valyl-tRNA synthetase, the rate of the process being approximately the same as for Val-$tRNA^{Val}$-C-C-A. Thr-$tRNA^{Val}$-C-C-A obtained by mischarging with threonyl-tRNA synthetase is hydrolyzed more than 30 times more rapidly by free valyl-tRNA synthetase than is Val-$tRNA^{Val}$-C-C-A (Figure 1a,b).

Thr-$tRNA^{Val}$-C-C-3′NH_2A, which is stable to hydrolysis by free enzyme (Figure 1d), does, however, take part in the AMP/PP independent hydrolysis under aminoacylating conditions (Figure 3f). This is to be compared with the Thr-$tRNA^{Val}$-C-C-3′dA case where the ester is unstable in both hydrolytic reactions (Figure 3d). Inspection of the reaction pathway leads one to expect that whereas aminoacylation and hydrolysis under aminoacylating conditions are opposing reactions, a third reaction which leads to stabilization of the esterified tRNA (such as transacylation from 2′-OH to 3′-NH_2) would, after a number of enzymatic cycles, including hydrolysis of the amino acid from the 2′-OH, result in a fully charged tRNA. This is what is observed for Thr-$tRNA^{Val}$-C-C-3′NH_2A while for Thr-$tRNA^{Val}$-C-C-3′dA no transacylation is possible and the overall result of aminoacylation then depends on the balance between the buildup and breakdown of Thr-$tRNA^{Val}$-C-C-3′dA, hence the variable and nonstoichiometric yield of this ester (Table I). The AMP/PP independent hydrolysis under aminoacylating conditions of Thr-$tRNA^{Val}$-C-C-A occurs as would be anticipated from the above arguments (Figure 3b) and demonstrates, in agreement with earlier findings (Fersht and Kaethner, 1976), that Thr-$tRNA^{Val}$-C-C-A is a functional intermediate during valyl-tRNA synthetase/threonine/$tRNA^{Val}$ interaction.

To substantiate the information obtained with modified tRNA, the behavior of two modifications of threonine was investigated. *O*-MeThr-$tRNA^{Val}$-C-C-N is formed with N = A, 3′dA, and 3′NH_2A in high yield under the catalytic influence of valyl-tRNA synthetase and these are all stable during AMP/PP independent hydrolysis under aminoacylation conditions, except for a slow hydrolysis in the presence of $tRNA^{Val}$-C-C-A (Figure 4a). This suggests that methylation of the OH group either affects the hydrolytic correction during which the OH group has a direct role or methylation causes a steric hindrance to the approach of the hydrolytic agent. With α-aminobutyric acid the possible direct interaction of the amino acid side chain is excluded and the steric effect is relieved. AMP/PP independent hydrolysis is observed during both $tRNA^{Val}$-C-C-A and $tRNA^{Val}$-C-C-3′dA charging, the rate of hydrolysis induced by $tRNA^{Val}$-C-C-A being the fastest obtained in this series of experiments.

These results lead to a relaxation in the structural requirements for correction. Bulky, nonhydroxylic β substituents on the amino acid lead to a stably charged tRNA, e.g., *O*-methylthreonine and, in the case of the *E. coli* enzyme, β-chloro-α-aminobutyric acid (Freundlich, 1967), from which only slow release of the amino acid occurs in the presence of the terminal ribose 3′-OH. On the other hand rapid correction takes place when the amino acid β carbon has an OH or is sterically unhindered, e.g., threonine and α-aminobutyric acid.

The results presented above may allow a number of conclusions regarding the molecular nature of the correction process carried out by valyl-tRNA synthetase to be drawn. Formation of a β-lactone intermediate through attack of the threonine OH on the α-carboxyl, which would seem possible in the light of interactions observed with valyl-tRNA synthetase from *E. coli* and from *A. californica* with cyclic amino acids (Chuang et al., 1967; Owens and Bell, 1970; Anderson and Fowden, 1970), can be ruled out. Nucleophilic hydrolysis of β-lactones at neutral pHs takes place by a bimolecular re-

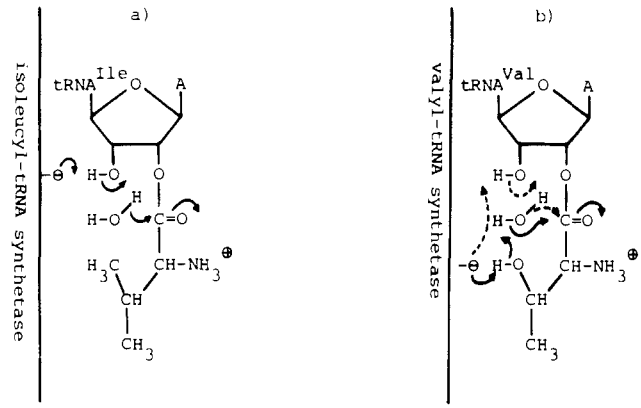

FIGURE 6: Diagrammatic representation of the proposed role of water in the hydrolytic correction of aminoacyl-tRNA by isoleucyl- or valyl-tRNA synthetase: (a) Val-tRNA^{Ile} as proposed by von der Haar and Cramer (1976); (b) Thr-tRNA^{Val} showing activation of H_2O via the threonine OH (-) and via the ribose 3′-OH (- - -) mechanistic pathways. The molecular nature of enzyme functional group - ⊖ is not defined.

action resulting in cleavage of the alkyl C–O bond (Ingold, 1953). As is the case for normal S_N2 reactions, if the center of nucleophilic attack is optically active, inversion of the configuration takes place. In the case of threonine, formation of a β-lactone from the natural L-threonine would, after hydrolysis, give the inverted L-allothreonine which is not a substrate for valyl-tRNA synthetase (in the PP exchange, at least; Bergmann et al., 1961; George and Meister, 1967). During AMP/PP independent hydrolysis under aminoacylating conditions when the ATP concentration is not limiting, such a conversion of threonine to allothreonine should lead to a plateau in the amount of AMP produced at a level equal to the initial threonine concentration. In Figure 4a we see that this is not the case. Threonine (10 μM) should consume 2% of the available ATP if it is converted to the allo form. Continued hydrolysis is observed.

Since in such a hydrolytic reaction it is trivially obvious that a hydroxyl function must participate, the alternative source of hydrolytic capacity is the aqueous environment of the active site. This water would then play a similar overall role as that proposed for it in the isoleucine/valine system (von der Haar and Cramer, 1976), with activation of the H_2O occurring through either the ribose 3′-OH, the threonine side-chain OH, or both, or in sterically favorable cases, e.g., with α-aminobutyric acid, by a more direct route.

Thus, the use of water as shown in Figure 6 in valyl-tRNA synthetase catalyzed hydrolysis of mischarged tRNA^{Val} bears overall similarities to the correction performed by isoleucyl-tRNA synthetase on Val-tRNA^{Ile} and provides a second example of chemical proofreading.

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