Sautière, P., Wouters-Tyrou, D., Laine, B., and Biserte, G. (1975), *Ciba Found. Symp. 28,* 77.
Van Holde, K. E., and Isenberg, I. (1975), *Acc. Chem. Res. 8,* 327.
Van Lente, F., Jackson, J. F., and Weintraub, H. (1975), *Cell 5,* 45.
Wangh, L., Ruiz-Carillo, A., and Allfrey, V. G. (1972), *Arch. Biochem. Biophys. 150,* 44.
Weintraub, H., Palter, K., and Van Lente, F. (1975), *Cell 6,* 85.
Williams, J., and Lowe, J. M. (1971), *Biochem. J. 121,* 203.

Hydrolytic Action of Aminoacyl-tRNA Synthetases from Baker's Yeast: "Chemical Proofreading" Preventing Acylation of $tRNA^{Ile}$ with Misactivated Valine†

Friedrich von der Haar* and Friedrich Cramer

ABSTRACT: Phe-$tRNA^{Phe}$-C-C-A, Val-$tRNA^{Val}$-C-C-A, and Ile-$tRNA^{Ile}$-C-C-A, which accept their amino acid on the 2′-OH of the 3′-terminal adenosine, are hydrolyzed readily by their aminoacyl-tRNA synthetase. If the 3′-terminal adenosine in these tRNAs is replaced by either 3′-deoxyadenosine or formycin, little if any hydrolysis can be observed. Correspondingly Ser-$tRNA^{Ser}$-C-C-A which accepts serine on the 3′-OH of the 3′-terminal adenosine is hydrolyzed by seryl-tRNA synthetase, whereas Ser-$tRNA^{Ser}$-C-C-2′dA and Ser-$tRNA^{Ser}$-C-C-F are not. Tyr-$tRNA^{Tyr}$-C-C-A and all modified Tyr-$tRNA^{Tyr}$-C-C-N, which can accept tyrosine on either the 2′-OH or the 3′-OH of the 3′-terminal adenosine, are not hydrolyzed by tyrosyl-tRNA synthetase. The data can be rationalized assuming that hydrolysis takes place only if the amino acid is bound to the nonaccepting OH and hence is not positioned at the amino acid binding site upon formation of the complex between aminoacyl-tRNA and aminoacyl-tRNA synthetase. In the formycin-carrying tRNA, the amino acid bound to the nonaccepting OH seems to be inaccessible to the enzymatic groups responsible for hydrolysis. Val-$tRNA^{Ile}$-C-C-3′dA and Ile-$tRNA^{Ile}$-C-C-3′dA cannot be hydrolyzed by isoleucyl-tRNA synthetase. Val-$tRNA^{Ile}$-C-C-A is hydrolyzed by the enzyme five times more rapidly than Ile-$tRNA^{Ile}$-C-C-A. Whereas Ile-$tRNA^{Ile}$-C-C-F is absolutely stable, Val-$tRNA^{Ile}$-C-C-F is hydrolyzed immediately. As shown by the earlier finding that valine misactivated by isoleucyl-tRNA synthetase cannot be permanently transferred to $tRNA^{Ile}$-C-C-A but to $tRNA^{Ile}$-C-C-3′dA, the 3′-OH is essential for preventing transfer of misactivated valine. It thus appears that valine is hydrolyzed off Val-$tRNA^{Ile}$-C-C-N if it is bound to the accepting 2′-OH in the binding site for isoleucine. A hypothesis is offered attempting to explain the experimental observations in mechanistic terms. We consider the hydrolytic action of the aminoacyl-tRNA synthetases as a general mechanism of "chemical proofreading" in the protein biosynthesis.

In protein biosynthesis amino acids are enzymatically activated (eq 1) and transferred to tRNA (eq 2) (Loftfield, 1972; Kisselev and Favorova, 1974; Söll and Schimmel, 1974). In these reactions, the required specificity is dependent entirely on the interaction of aminoacyl-tRNA synthetase with its specific amino acid (aa[1]) and its specific tRNA.

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

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

In addition to the reactions described in eq 1 and 2, aminoacyl-tRNA synthetases can perform a hydrolytic reaction; aminoacyl-tRNAs are in most cases hydrolyzed on interaction with their enzyme (eq 3). This reaction is known in the literature as AMP/PP independent hydrolysis.

$$\text{aa-tRNA} + \text{enzyme} \rightarrow \text{aa} + \text{tRNA} + \text{enzyme} \quad (3)$$

It has been speculated, whether this hydrolysis might be functional in a control step in order to increase the specificity of the reactions described in eq 1 and 2 (Söll and Schimmel, 1974; Yaniv and Gros, 1969; Lagerkvist et al., 1966; Schreier and Schimmel, 1972; Bonnet and Ebel, 1972; Sourgoutchov et al., 1974), because the specificity of the aminoacyl-tRNA synthetases with respect to amino acids is no one absolute value (Loftfield, 1972). One of the best investigated irregularities is misactivation of valine by isoleucyl-tRNA synthetase from *Escherichia coli* or from baker's yeast according to eq 1, yielding [E^{Ile}·Val-AMP] (Söll and Schimmel, 1974, see section IVD; Bergmann et al., 1961; Loftfield and Eigner, 1965; Baldwin and Berg, 1966; von der Haar and Cramer, 1975). Transfer of this misactivated valine to $tRNA^{Ile}$-C-C-A is prevented by a hydrolytic reaction which leads to the liberation of valine from [E^{Ile}·Val-AMP] on treatment with $tRNA^{Ile}$-C-C-A (eq 4) (Baldwin and Berg, 1966), thus correcting the previous misactivation.

$$[E^{Ile} \cdot \text{Val-AMP}] + tRNA^{Ile}\text{-C-C-A} \rightarrow \text{Val} + \text{AMP} + E^{Ile} + tRNA^{Ile}\text{-C-C-A} \quad (4)$$

Previously we have found a strict specificity for the position

† From Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, 3400 Göttingen, Germany. *Received February 11, 1976.*

[1] Abbreviations used: aa, amino acid; E, enzyme; F, formycin (8-aza-9-deazaadenosine); Xxx-$tRNA^{Yyy}$-C-C-N, tRNA specific for amino acid Yyy, fitted with terminal nucleoside N, and aminoacylated with Xxx- at the 2′- or 3′-position as determined by the terminal nucleoside N and the corresponding enzyme; [E^{Xxx}·Yyy-AMP], binary complex of the aminoacyl-tRNA synthetase specific for Xxx and the aminoacyl adenylate of the amino acid Yyy; Tris, tris(hydroxymethyl)aminomethane; uv, ultraviolet; DEAE, diethylaminoethyl; PEI, poly(ethylenimine).

of the accepting hydroxyl group in the 3′-terminal ribose of a tRNA (Cramer et al., 1975; Sprinzl et al., 1973); for example, $tRNA^{Phe}$, $tRNA^{Ile}$, and $tRNA^{Val}$ accept their amino acid on the 2′-hydroxyl group, whereas $tRNA^{Ser}$ is aminoacylated on the 3′-hydroxyl group and $tRNA^{Tyr}$ can accept its amino acid on either the 3′- or the 2′-hydroxyl group.

We also have recently observed that misactivated valine is readily transferred to $tRNA^{Ile}$-C-C-3′dA, a $tRNA^{Ile}$ which lacks the 3′-hydroxyl group of the 3′-terminal ribose (von der Haar and Cramer, 1975), indicating some kind of involvement of the nonaccepting hydroxyl group in the corrective hydrolysis of [E^{Ile}·Val-AMP].

In continuation of these studies we could, therefore, hope to answer the following questions: (a) is the accepting or nonaccepting hydroxyl group required in the AMP/PP independent hydrolysis (eq 3), and (b) what is the detailed hydrolytic mechanism of the correction step preventing valylation of $tRNA^{Ile}$ by [E^{Ile}·Val-AMP] (eq 4)?

We therefore have now investigated the hydrolysis of a series of aminoacyl-tRNAs with a modified 3′-terminal adenosine in the phenylalanine, valine, serine, tyrosine, and isoleucine systems and do indeed find that the nonaccepting hydroxyl group of the tRNA is instrumental in the hydrolytic reaction.

Experimental Section

Materials

$tRNA^{Phe}$-C-C, $tRNA^{Ser}$-C-C, and $tRNA^{Tyr}$-C-C were purified from unfractionated tRNA from baker's yeast (supplied by Boehringer, Mannheim, Germany) as described (Schneider et al., 1972). $tRNA^{Val}$-C-C and $tRNA^{Ile}$-C-C were isolated by an analogous procedure. Incorporation of the nucleotides 3′dAMP, 2′dAMP, formycin, and 3′-deoxy-3′-aminoadenosine 5′-phosphate with tRNA-nucleotidyl transferase (EC 2.7.7.21) followed the general procedure published (Cramer et al., 1975; Sprinzl et al., 1973; Maelicke et al., 1974).

Phenylalanyl- (EC 6.1.1.20), valyl- (EC 6.1.1.9), seryl- (EC 6.1.1.11), tyrosyl- (EC 6.1.1.1), and isoleucyl-tRNA synthetases (EC 6.1.1.5) were all purified by affinity elution (von der Haar, 1973; Faulhammer and Cramer, 1974). [^{14}C]ATP of specific activity 44 mCi/mmol was purchased from Radiochemical Centre (Amersham, England). Under the experimental conditions, 1 nmol corresponds to 53 000 cpm. Amino acids of Stanstar grade (50 mCi/mmol) were a product of Schwarz Bioresearch (Orangeburg, N.Y.); 60 000 cpm correspond to 1 nmol. All other salts and reagents were of analytical grade commercially available.

Methods

(*a*) *Production of AMP under Aminoacylation Conditions.* The assay mixture contained 150 mM Tris-HCl (pH 7.6), 150 mM KCl, 10 mM $MgSO_4$, 1 mM amino acid, 0.5 mM [^{14}C]ATP, and 4.8 μM tRNA-C-C-N. The reaction was performed in 50 μl at 36 °C. The amount of enzyme was chosen such that for the calculation of initial velocities the first two time values were in the linear range. After the time values given in the figures, 1-μl aliquots were withdrawn from the reaction mixture and spotted onto PEI-cellulose/uv_{254} plates (Macherey and Nagel, Düren, Germany). Unlabeled ATP, ADP, and AMP were applied to the plates at the origin prior to use. The nucleotides were separated by ascending chromatography for 40 min with 0.75 M potassium phosphate buffer (pH 3.5). After drying the spots were marked under a uv lamp. Nucleotide containing spots were cut out and prepared for liquid scintillation counting as described (Schlimme et al., 1969).

As outlined in Results each hydrolysis of an aminoacyl-tRNA molecule in the aminoacylation assay will cause the formation of one molecule of AMP. The hydrolysis of aminoacyl-tRNA will, however, be unequivocally indicated by the AMP formation only if no other generation of AMP occurs. Therefore ATP, ADP, and AMP were measured simultaneously in the absence of both amino acid and tRNA as well as in the absence of only tRNA and in neither case was AMP formed. Therefore both ATP and the [E·aa-AMP] complex formed in the presence of enzyme, ATP, and amino acid were stable under the reaction conditions.

The level of ADP, which is present in an amount of 5–20% in different commercial preparations of [^{14}C]ATP, remained constant in these experiments. Testing partially purified aminoacyl-tRNA synthetase preparations, we regularly observed an increase of ADP as well as of AMP during the reaction, indicating that unspecific attack on ATP by enzymatic contaminants is reflected by ADP formation. Hence the stability of ATP in experiments with purified aminoacyl-tRNA synthetases is a good indication for the absence of such contaminant enzymes.

(*b*) *Hydrolysis of Aminoacyl-tRNA-C-C-N with Free Enzyme (AMP/PP Independent).* The assay mixture contained 150 mM Tris-HCl (pH 7.6), 150 mM KCl, 10 mM $MgSO_4$, and 2.5 μM [^{14}C]aminoacyl-tRNA in addition to the enzyme specified in the figures. The reactions were performed at 36 °C in 200 μl of assay mixture. After the times specified in the figures, 20 μl aliquots were withdrawn and prepared for liquid scintillation counting as described (Schlimme et al., 1969). Amount of enzyme was chosen according to the criteria given under a. Spontaneous hydrolysis was determined in absence of enzyme under otherwise identical conditions.

(*c*) *Calculation of Turnover Numbers.* Because of the large differences in molecular weight for the enzymes investigated, turnover numbers calculated from the initial velocities instead of specific activities are given in the tables. For this purpose molecular weights of 260 000 for phenylalanyl-tRNA synthetase (Fasiolo et al., 1974), 120 000 for valyl- (Rymo et al., 1972) and seryl-tRNA synthetase (Heider et al., 1971), and 45 000 for tyrosyl-tRNA synthetase (Beikirch et al., 1972) were used. The molecular weight for isoleucyl-tRNA synthetase from baker's yeast has not been described. However, during sodium dodecyl sulfate gel electrophoresis it migrates as a single band just in front of valyl-tRNA synthetase. For this reason a molecular weight of 115 000 was taken. Calculation was performed assuming a single reactive site for all five enzymes, which is in agreement with the literature for valyl- (Rymo et al., 1972), isoleucyl- (unpublished), and tyrosyl-tRNA synthetase (Beikirch et al., 1972). For phenylalanyl-tRNA synthetase (Fasiolo et al., 1974) and seryl-tRNA synthetase (Engel et al., 1972), two binding sites have been postulated but it is not certain whether these are equivalent reactive sites (Fasiolo et al., 1974; Pingoud et al., 1973). Therefore one must bear in mind that the numerical values given for these two enzymes may be wrong by a factor of two. However, since the interpretation given is based on qualitative rather than on quantitative aspects, this uncertainty does not present difficulties in discussing the results.

(*d*) *Preparation of* [^{14}C]*Aminoacyl-tRNA-C-C-N with Cognate Amino Acids.* Aminoacylation was performed in 1 ml of assay mixture containing 150 mM Tris-HCl (pH 7.6), 50 mM KCl, 10 mM $MgSO_4$, 1.5 mM ATP, 60 μM ^{14}C-labeled amino acids, and 7.5 μM tRNA-C-C-N. Prior to pre-

TABLE I: Hydrolysis with Free Enzyme of Aminoacyl-tRNA-C-C-N.

Enzyme	Aminoacyl-tRNA	Spontaneous Hydrolysis ($nmol^{-1}$ min^{-1} $l.^{-1}$)	Enzymatic Hydrolysis[a] Turnover Number (min^{-1})
Phe-tRNA synthetase	Phe-tRNA^{Phe}-C-C-A	80	0.67
	Phe-tRNA^{Phe}-C-C-F	7.5	0.06
	Phe-tRNA^{Phe}-C-C-3′dA	6.8	0.07
Val-tRNA synthetase	Val-tRNA^{Val}-C-C-A	<1	0.77
	Val-tRNA^{Val}-C-C-F	<1	<0.03
	Val-tRNA^{Val}-C-C-3′dA	<1	0.07
Ser-tRNA synthetase	Ser-tRNA^{Ser}-C-C-A	62.5	0.62
	Ser-tRNA^{Ser}-C-C-F	<1	<0.03
	Ser-tRNA^{Ser}-C-C-2′dA	<1	<0.03
Tyr-tRNA synthetase	Tyr-tRNA^{Tyr}-C-C-A	124	<0.02
	Tyr-tRNA^{Tyr}-C-C-2′dA	<1	<0.02
	Tyr-tRNA^{Tyr}-C-C-3′dA	<1	0.06
Ile-tRNA synthetase	Ile-tRNA^{Ile}-C-C-A	12.5	1.8
	Ile-tRNA^{Ile}-C-C-F	<1	<0.03
	Ile-tRNA^{Ile}-C-C-3′dA	<1	<0.03
	Val-tRNA^{Ile}-C-C-A	12.5	9.3
	Val-tRNA^{Ile}-C-C-F	<1	35.6
	Val-tRNA^{Ile}-C-C-3′dA	<1	<0.03
	Phe-tRNA^{Ile}-C-C-A	142	1.6

[a] All values have been corrected for spontaneous hydrolysis.

parative work, the amount of enzyme needed was determined in such a way that maximum aminoacylation was obtained after 5 min and was kept constant for a further period of 20 min. Preparative reaction was then carried out for 10 min at 36 °C. The reaction mixture was diluted with 1 volume of water and poured over 1 ml of DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) which was equilibrated with 0.02 M sodium acetate buffer (pH 5.2).

All assay components except tRNA were released from the column by washing with 10 ml of the equilibrating buffer containing 0.5 M NaCl. Aminoacyl-tRNA was released from the column with the same buffer containing 1 M NaCl. The tRNA-containing solution was concentrated to 2 ml on a rotary evaporator at room temperature and desalted by passing it over a 1.5 × 25 cm Bio-Gel P-2 column (Bio-Rad, Richmond, Va.). Finally the desalted solution was again concentrated to an appropriate volume. The preparation obtained in this way could be stored at −18 °C without any release of amino acid from the tRNA for several months. Repeated freezing and thawing did not destroy the aminoacyl-tRNA linkage.

(*e*) *Misaminoacylation of tRNA^{Ile}-C-C-N with Valine.* Preparation of Val-tRNA^{Ile}-C-C-N was performed in 1 ml of assay mixture containing 10 mM Tris-HCl (pH 9.0), 8 mM $MgSO_4$, 0.5 mM ATP, 60 μM [^{14}C]valine, and 7.5 μM tRNA^{Ile}-C-C-N. Valyl-tRNA synthetase (150 μg) was added and reaction was maintained for 10 min at 36 °C. The working-up procedure was essentially the same as described in section d.

(*f*) *Misaminoacylation of tRNA^{Ile}-C-C-N with Phenylalanine.* Reaction conditions were the same as for e, except that valine was replaced by phenylalanine and the assay mixture was made 20% in dimethyl sulfoxide (v/v). Phenylalanyl-tRNA synthetase (225 μg) was used for this reaction.

Results

(*I*) *Hydrolysis of Preformed Aminoacyl-tRNA with Free Enzyme* (*AMP/PP Independent*) (*Equation 3*). Hydrolyses of the 2′-hydroxyl group accepting aa-tRNA-C-C-N specific for valine, isoleucine, and phenylalanine all show qualitatively (Figure 1a,b,e,f) and quantitatively (Table I) the same behavior. aa-tRNA-C-C-A is significantly hydrolyzed, whereas hydrolyses of aa-tRNA-C-C-3′dA and aa-tRNA-C-C-F are almost negligible. In the 3′-hydroxyl group accepting serine system Ser-tRNA^{Ser}-C-C-A is hydrolyzed whereas Ser-tRNA^{Ser}-C-C-2′dA and Ser-tRNA^{Ser}-C-C-F are stable (Figure 1c). In the tyrosine system, in which both the 2′- and the 3′-hydroxyl group can be tyrosylated, none of the Tyr-tRNA^{Tyr}-C-C-N's including Tyr-tRNA^{Tyr}-C-C-A is hydrolyzed (Figure 1d).

In the isoleucine system Phe-tRNA^{Ile}-C-C-A was also prepared and subjected to isoleucyl-tRNA synthetase dependent hydrolysis. From Table I can be seen that this misaminoacylated Phe-tRNA^{Ile}-C-C-A is hydrolyzed as readily as the normal Ile-tRNA^{Ile}-C-C-A. This is in contrast to the *E. coli* system in which Phe-tRNA^{Ile}-C-C-A was not hydrolyzed at all (Eldred and Schimmel, 1972). An attempt to misaminoacylate tRNA^{Ile}-C-C-F and tRNA^{Ile}-C-C-3′dA with phenylalanine for further comparison has failed so far.

On substitution of isoleucine (the normal amino acid for tRNA^{Ile}) by valine (the amino acid misactivated in the isoleucine system), the following results were obtained (Figure 1e,f and Table I). The turnover number for hydrolysis of Val-tRNA^{Ile}-C-C-A is increased fivefold compared with Ile-tRNA^{Ile}-C-C-A. This result is quantitatively but not qualitatively different from that reported for isoleucyl-tRNA synthetase from *E. coli* (Eldred and Schimmel, 1972), where Val-tRNA^{Ile}-C-C-A was hydrolyzed 1000 times faster than Ile-tRNA^{Ile}-C-C-A. Val-tRNA^{Ile}-C-C-3′dA is stable, as is Ile-tRNA^{Ile}-C-C-3′dA. A dramatic difference is found for Val-tRNA^{Ile}-C-C-F as compared with Ile-tRNA^{Ile}-C-C-F. Whereas Ile-tRNA^{Ile}-C-C-F is absolutely stable against hydrolysis by isoleucyl-tRNA synthetase, Val-tRNA^{Ile}-C-C-F is hydrolyzed with the highest turnover number observed in all our experiments.

(*II*) *AMP/PP Independent Hydrolysis of Aminoacyl-tRNA-C-C-N with* [*E·aa-AMP*] *Complex.* The AMP/PP

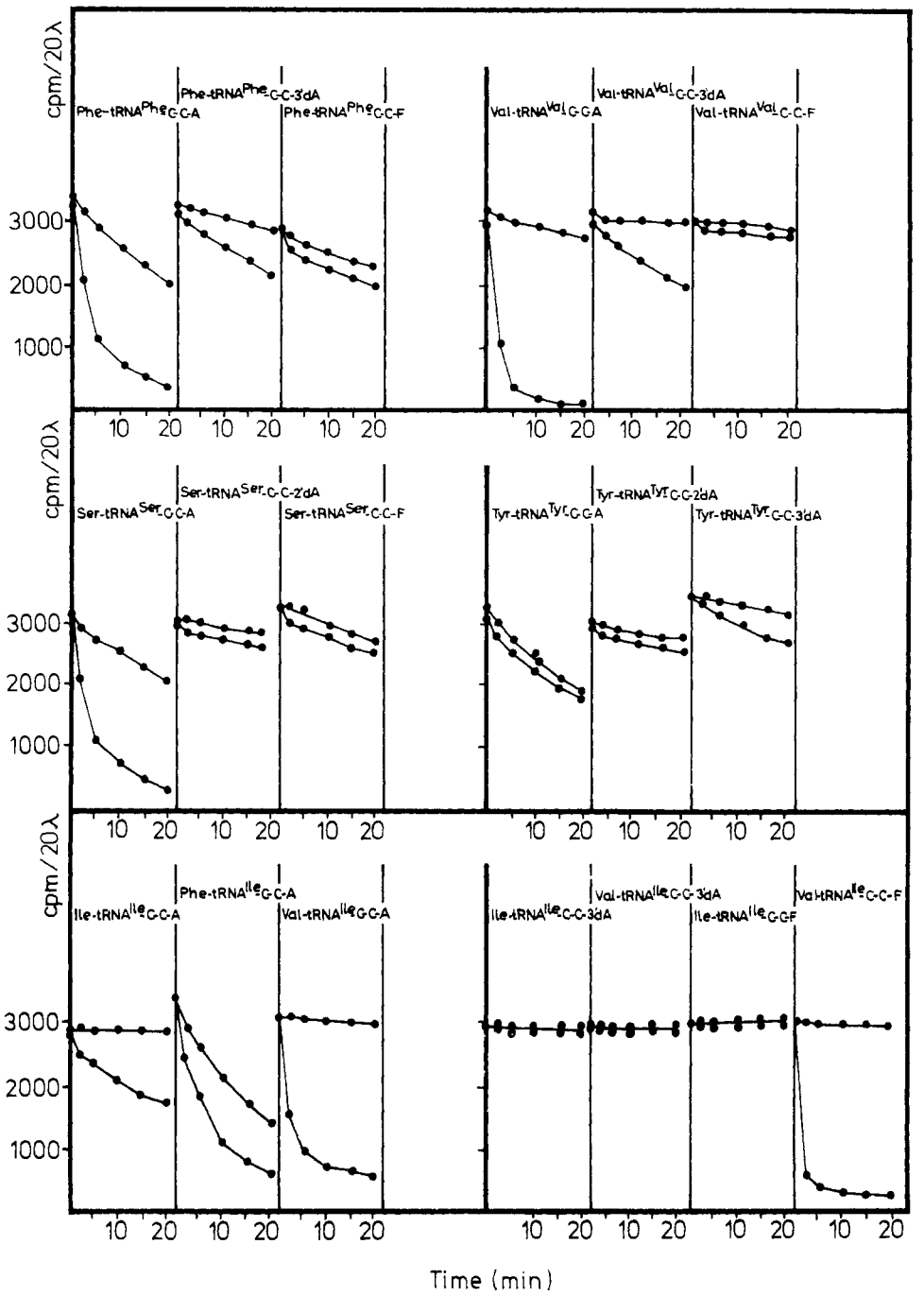


FIGURE 1: Hydrolysis of aminoacyl-tRNA-C-C-N with free enzyme (AMP/PP independent). Data were obtained from the assay described in Methods. The upper lines give the spontaneous hydrolysis of aminoacyl-tRNA determined in the absence of enzyme. The lower lines give the hydrolysis in the presence of enzyme. Hence the enzyme-induced hydrolysis is the difference between lower and upper lines. The molarities of the respective aminoacyl-tRNA synthetases were 0.6, 1.6, 0.6, 1.6, 0.07, and 0.07 μM. (a) (above, left) Phenylalanine system; (b) (above, right) valine system; (c) (middle, left) serine system; (d) (middle, right) tyrosine system; (e) (below, left) isoleucine system with various amino acids and 3'-terminal adenosine; (f) (below, right) isoleucine system with various amino acids and modified 3'-terminal adenosine.

independent hydrolysis of aminoacyl-tRNA is usually investigated with free enzyme and preformed aa-tRNA (see section I). An important question, however, is whether this type of hydrolysis also takes place with the [E·aa-AMP] complex, which is the predominant species present during the aminoacylation of tRNA in presence of enzyme, ATP, and amino acid. An assay to investigate this reaction can be based on the following rationale. If hydrolysis of aa-tRNA with [E·aa-AMP] takes place, then the system described in eq 1 and 2 in the introduction is not at rest after all the tRNA is aminoacylated. Instead aa-tRNA is continuously hydrolyzed generating free tRNA, which subsequently is reaminoacylated. As a consequence, one AMP is formed for each aa-tRNA hydrolyzed (eq 5) in addition to the 1:1 stoichiometry of AMP formation per aminoacylated tRNA according to eq 1 and 2.

$$[E \cdot aa\text{-}AMP] + aa\text{-}tRNA \rightarrow aa + [E \cdot tRNA \cdot aa\text{-}AMP] \rightarrow aa\text{-}tRNA + E + AMP \quad (5)$$

To investigate this reaction we therefore substituted the radioactively labeled amino acid usually present in the aminoacylation assay by radioactively labeled ATP and determined the formation of labeled AMP.

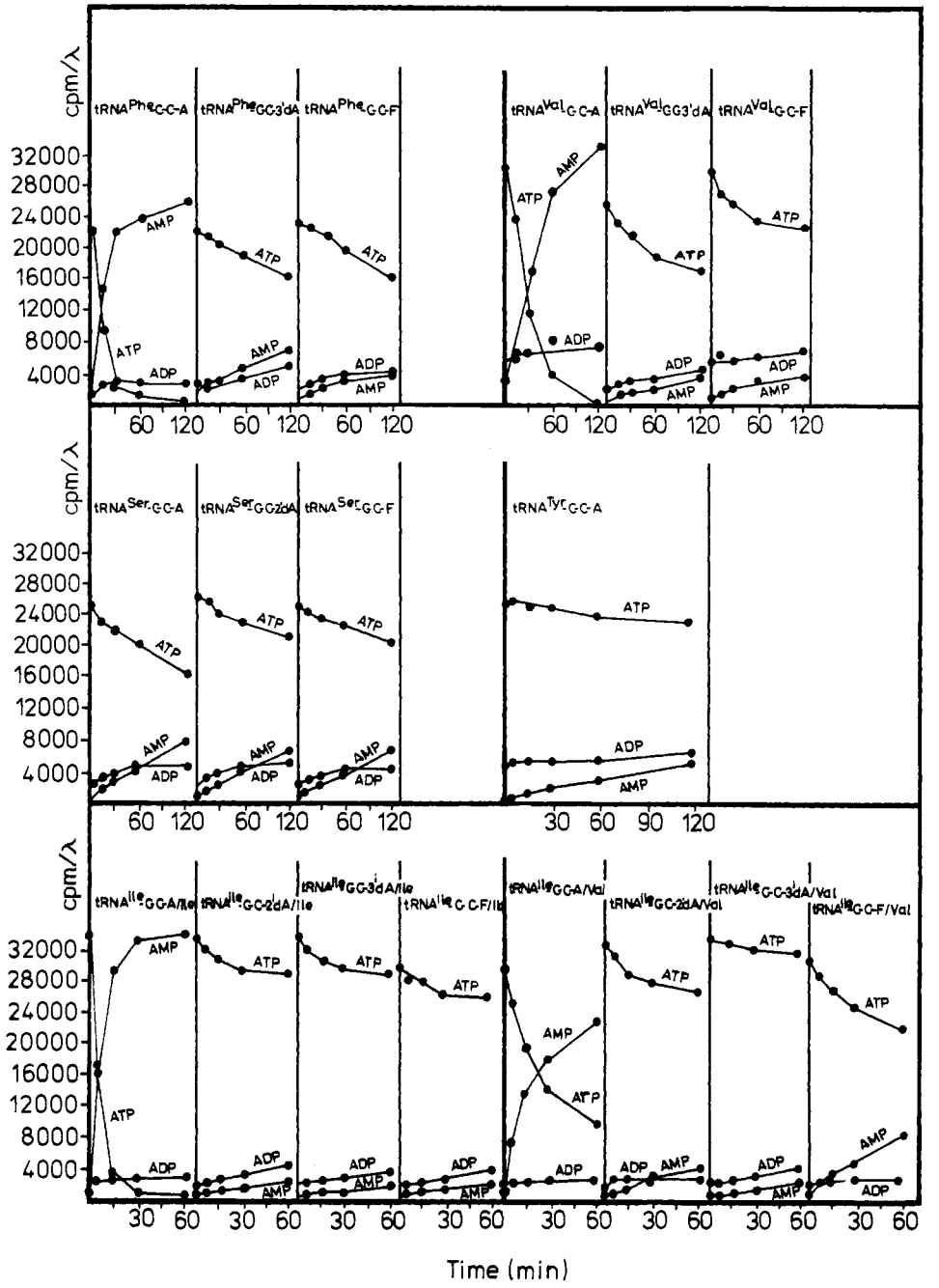


FIGURE 2: AMP formation under conditions of aminoacylation. Data were obtained from the assay described in Methods. The molarities of the respective aminoacyl-tRNA synthetases were 1.8, 8.7, 2.7, 8.0, 3.5, and 3.5 μM. (a) (above, left) Phenylalanine system; (b) (above, right) valine system; (c) (middle, left) serine system; (d) (middle, right) tyrosine system; (e) (below, left) isoleucine system; (f) (below, right) isoleucine system with valine substituted for isoleucine.

Whereas the presence of tRNA^{Phe}-C-C-A in the aminoacylation assay leads to very rapid AMP formation, this reaction is rather slow with tRNA^{Phe}-C-C-3′dA and tRNA^{Phe}-C-C-F (Figure 2a). Since a 100-fold excess of ATP over tRNA was used and since all the ATP was transformed into AMP, every Phe-tRNA^{Phe}-C-C-A was hydrolyzed and reaminoacylated 100 times within about 15 min.

Qualitatively the same is found for the valine and isoleucine systems. The tRNA-C-C-A leads to AMP formation whereas both tRNA-C-C-3′dA and tRNA-C-C-F do not (Figure 2b,e). In comparison with the 2′-hydroxyl group acceptors specific for phenylalanine, valine, and isoleucine, the 3′-hydroxyl group accepting tRNA^{Ser} behaves differently. For this system all three substrates, tRNA^{Ser}-C-C-A, tRNA^{Ser}-C-C-F, and tRNA^{Ser}-C-C-2′dA, lead to slow AMP formation (Figure 2c). tRNA^{Tyr}-C-C-A as well as all modified tRNA^{Tyr} tested do not show any AMP formation exceeding the 1:1 stoichiometry corresponding to the first round of aminoacylation (Figure 2d).

Numerical values for the turnover numbers of AMP formation derived from these experiments are compared with the turnover numbers of aminoacyl-tRNA formation (Table II). With the exception of the isoleucine system, formation of AMP is at least 30-fold slower than aminoacylation. Hence hydrolysis of aminoacyl-tRNA must be the rate-limiting step and AMP formation does, therefore, indicate the rate of hydrolysis of aminoacyl-tRNA. In the isoleucine system the ratio of aminoacylation vs. hydrolysis is especially low and this may

TABLE II: AMP Formation under Conditions of Aminoacylation.

Enzyme	Amino Acid	tRNA	Turnover Number (min^{-1})	
			Aminoacylation[a]	AMP Formation
Phe-tRNA synthetase	Phe	$tRNA^{Phe}$-C-C-A	300.00	10.05
		$tRNA^{Phe}$-C-C-F	10.50	0.35
		$tRNA^{Phe}$-C-C-3′dA	240.00	0.70
Val-tRNA synthetase	Val	$tRNA^{Val}$-C-C-A	49.00	1.00
		$tRNA^{Val}$-C-C-F	23.00	0.08
		$tRNA^{Val}$-C-C-3′dA	13.00	0.08
Ser-tRNA synthetase	Ser	$tRNA^{Ser}$-C-C-A	16.30	0.70
		$tRNA^{Ser}$-C-C-F	11.00	0.55
		$tRNA^{Ser}$-C-C-2′dA	8.50	0.55
Tyr-tRNA synthetase	Tyr	$tRNA^{Tyr}$-C-C-A	537.00	0.04
Ile-tRNA synthetase	Ile	$tRNA^{Ile}$-C-C-A	62.00	17.00
		$tRNA^{Ile}$-C-C-F	34.00	0.05
		$tRNA^{Ile}$-C-C-3′dA	45.00	0.05
		$tRNA^{Ile}$-C-C-2′dA	0	0.05
	Val	$tRNA^{Ile}$-C-C-A	0	8.30
		$tRNA^{Ile}$-C-C-F	0	1.50
		$tRNA^{Ile}$-C-C-3′dA	2.90	0.05
		$tRNA^{Ile}$-C-C-2′dA	0	0.20

[a] Values determined in the usual way (Schlimme et al., 1969) by replacing [^{14}C]ATP by [^{14}C]phenylalanine in the AMP forming assay under otherwise identical conditions.

be taken as an indication that the hydrolytic capacity is especially well developed in the isoleucine system. Comparing turnover numbers of hydrolysis of preformed aa-tRNA with free enzyme to hydrolysis with [E·aa-AMP] complex, one finds two obvious differences. In the serine and valine systems, the turnover numbers are about the same in both assay systems, whereas in the phenylalanine and isoleucine systems the turnover numbers for hydrolysis with free enzyme are about tenfold lower than for hydrolysis with [E·aa-AMP].

(III) Hydrolysis of [E^{Ile}·Val-AMP] with $tRNA^{Ile}$-C-C-N. Hydrolysis of [E^{Ile}·Val-AMP] can be followed experimentally using the same assay as described in section II since, similarly, in this case any hydrolysis step leads to the formation of one AMP. With this system the following results were obtained (Figure 2f): (a) $tRNA^{Ile}$-C-C-A as well as $tRNA^{Ile}$-C-C-F leads to AMP formation; (b) $tRNA^{Ile}$-C-C-2′dA, which is not a substrate, and $tRNA^{Ile}$-C-C-3′dA, which is a substrate, do not show any reaction. Comparing the turnover numbers (Table II) for AMP formation with valine to those obtained with isoleucine, one can see that AMP formation with valine and $tRNA^{Ile}$-C-C-A is about half as fast as with isoleucine. The ratio of turnover numbers for $tRNA^{Ile}$-C-C-A/$tRNA^{Ile}$-C-C-F is 5.5 for valine but >340 for isoleucine.

(IV) Valylation of $tRNA^{Ile}$-C-C-N by [E^{Ile}·Val-AMP]. As reported earlier (von der Haar and Cramer, 1975) misactivated valine is transferred to $tRNA^{Ile}$-C-C-3′dA. Within all our modified $tRNA^{Ile}$-C-C-N tested the only other $tRNA^{Ile}$, which accepted misactivated valine, was $tRNA^{Ile}$-C-C-3′-deoxy-3′amino-A, which carries an amino group instead of a hydroxyl group at its 3′-position on the terminal adenosine (Fraser and Rich, 1973).

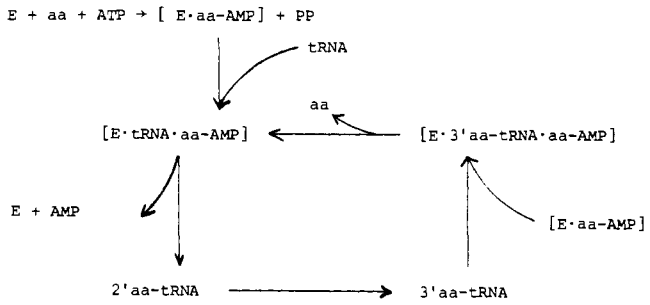
Discussion

I. Specificity of AMP/PP Independent Hydrolysis with Respect to the 2′- or 3′-Hydroxyl Group of the 3′-Terminal Ribose of Aminoacyl-tRNA. The tRNA/enzyme systems studied in this work can be classified in the three groups: $tRNA^{Phe}$, $tRNA^{Val}$, and $tRNA^{Ile}$, belong to the family of tRNAs which accept their amino acid on the 2′-hydroxyl group of the terminal adenosine, whereas $tRNA^{Ser}$ is a 3′-hydroxyl group acceptor and $tRNA^{Tyr}$ can accept its amino acid on either the 3′- or 2′-hydroxyl group (Cramer et al., 1975).

With respect to these families it is obvious (Figure 1 and Table I) that the nonaccepting hydroxyl is required for the AMP/PP independent hydrolysis. For example, for $tRNA^{Phe}$ the rate of aminoacylation is about the same with $tRNA^{Phe}$-C-C-A and $tRNA^{Phe}$-C-C-3′dA (turnover numbers 300 and 240), whereas the hydrolysis is decreased to $^1/_{15}$th when the 3′-hydroxyl group is lacking. Similar results are obtained with the 2′-aminoacylating system specific for valine and isoleucine. In contrast, the 3′-aminoacylating system of serine requires the 2′-hydroxyl group of $tRNA^{Ser}$ for its hydrolytic action, although in this case the effect is not so pronounced with the [E^{Ser}·Ser-AMP] complex but can be clearly seen in the hydrolysis with free enzyme (Table II). Interestingly, the tyrosine system, which according to our previous results (Cramer et al., 1975) is unspecific with respect to the accepting position, does not show at all the phenomenon of AMP/PP independent hydrolysis. This suggests that the phenomena of 2′ or 3′ specificity and enzymatic hydrolysis are directly connected.

As a rationale for these findings, we would like to present the following mechanism: the amino acid is hydrolyzed off the tRNA only if it is positioned on the nonaccepting hydroxyl group (or vice versa, it is protected if bound to the accepting hydroxyl group and hence positioned in the original amino acid binding site on the enzyme). The sequence of events for a 2′-accepting tRNA on interaction with [E·aa-AMP] is outlined in Scheme I. A tRNA is aminoacylated on the 2′-hydroxyl group. After it is released from the enzyme surface, the amino acid rapidly interchanges between the 2′- and the 3′-hydroxyl groups (Wolfenden et al., 1964). The tRNA carrying its amino acid on the 3′-hydroxyl group can again be bound by the [E·aa-AMP] complex. The amino acid is then released and the free tRNA is reaminoacylated.

Scheme I



tRNAs carrying a formycin do not exhibit the hydrolytic action in spite of the fact that they also possess a cis diol on their 3′ terminus. Since interchange of ester between neighboring hydroxyl groups is also observed for cis diols not fixed conformationally in a ring structure (Wolfenden et al., 1964; McLaughlin and Ingram, 1965; Griffin et al., 1966), it seems unlikely that this interchange could be prevented because of differences between adenosine and formycin in ribose puckering (Prusiner et al., 1973). Other cases have been described (Baksht et al., 1975; Shiloach et al., 1975) in which aminoacyl-tRNA-C-C-F qualitatively but not quantitatively behaves identically with aminoacyl-tRNA-C-C-A in enzymatic reactions. A plausible explanation for the inability of hydrolysis of aa-tRNA-C-C-F may be that the amino acid ester on the 3′-hydroxyl group occupies a position which is not accessible for the enzymatic groups catalyzing the hydrolysis in a 2′-accepting system.

II. Correction of Misactivation of Valine in the Isoleucine System. Two questions now arise with respect to the correction of the misactivation of valine: (a) Must valine be transferred onto $tRNA^{Ile}$ prior to corrective hydrolysis and (b) if it is transferred, does hydrolysis occur from the 3′- or from the 2′-hydroxyl group?

Three pieces of evidence support the idea that valine is transferred to $tRNA^{Ile}$. Firstly, we observe a ready hydrolysis of Val-$tRNA^{Ile}$-C-C-A and Val-$tRNA^{Ile}$-C-C-F with free enzyme, indicating that AMP is not necessary to perform the correction step.

Secondly, the rate of this hydrolysis is more rapid with Val-$tRNA^{Ile}$-C-C-A and Val-$tRNA^{Ile}$-C-C-F than with Ile-$tRNA^{Ile}$-C-C-A when measured directly, whereas in the AMP forming test the rate of hydrolysis of Val-$tRNA^{Ile}$-C-C-A (or F) by [E^{Ile}·Val-AMP] is reduced and that of Ile-$tRNA^{Ile}$-C-C-A by [E^{Ile}·Ile-AMP] is enhanced. These observations are easily explained assuming that the transfer of valine to $tRNA^{Ile}$-C-C-N is slow and is followed by a fast hydrolysis from the tRNA molecule. The slow transfer is then the rate-limiting step in the AMP forming hydrolysis with [E^{Ile}·Val-AMP], whereas the fast hydrolysis off the tRNA is the rate-limiting step during reaction of Val-$tRNA^{Ile}$-C-C-N with free enzyme.

Thirdly, valylation of $tRNA^{Ile}$-C-C-3′dA and $tRNA^{Ile}$-C-C-3′-deoxy-3′-amino-A demonstrates that there exists no specific barrier for the transfer of misactivated valine to a $tRNA^{Ile}$-C-C-N.

From which hydroxyl of the 3′-terminal adenosine of $tRNA^{Ile}$-C-C-N is the valine now hydrolyzed? To decide this question the different behavior of Val-$tRNA^{Ile}$-C-C-F and Ile-$tRNA^{Ile}$-C-C-F may be considered. Ile-$tRNA^{Ile}$-C-C-F is stable when isoleucine is fixed to the 2′-hydroxyl group because the amino acid is protected by its being positioned in the binding site. Ile-$tRNA^{Ile}$-C-C-F (in contrast to Ile-$tRNA^{Ile}$-C-C-A) is also stable if the amino acid is fixed to the 3′-hydroxyl group, probably because of the different ribose puckering in adenosine and formycin (see Discussion, section I). Ile-$tRNA^{Ile}$-C-C-A and Phe-$tRNA^{Ile}$-C-C-A react with identical rates during isoleucyl-tRNA synthetase catalyzed hydrolysis. One should, therefore, also expect that valine would not show an essentially different behavior during reaction with isoleucyl-tRNA synthetase if fixed to the nonaccepting 3′-hydroxyl group of $tRNA^{Ile}$-C-C-N. Val-$tRNA^{Ile}$-C-C-F does, however, show the fastest hydrolysis in this system (20 times faster than Ile-$tRNA^{Ile}$-C-C-A, for example). One, therefore, has to conclude that valine is hydrolyzed from Val-$tRNA^{Ile}$-C-C-F while the amino acid is fixed to the accepting 2′-hydroxyl group. Hence, whereas isoleucine is protected against hydrolysis in the isoleucine binding site, the misactivated amino acid is labile in the isoleucine binding site.

Experimental evidence, which has to be taken into consideration for drawing a mechanistic picture of the correction step after misactivation of valine, is in summary:

(a) Valine, misactivated by isoleucyl-tRNA synthetase, is transferred to $tRNA^{Ile}$ prior to correction.

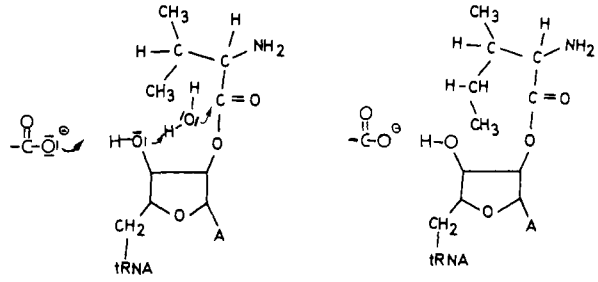
(b) The valine-tRNA ester bond is hydrolyzed prior to release of Val-$tRNA^{Ile}$-C-C-N while valine is remaining in the isoleucine binding site.

(c) To perform this hydrolysis the 3′-hydroxyl group of the 3′-terminal ribose of $tRNA^{Ile}$-C-C-N is essential (von der Haar and Cramer, 1975).

(d) The enzyme must be able to recognize the absence of the methyl group in valine compared with isoleucine.

A hypothesis attempting to describe the data in mechanistic terms is outlined in Scheme II. The misactivated valine is

Scheme II



transferred to $tRNA^{Ile}$-C-C-A. A water molecule may then be placed isosterically in the space of the missing methyl group of the normal substrate, isoleucine. This water molecule can be activated by a negatively charged group of the enzyme via the 3′-hydroxyl group of the $tRNA^{Ile}$-C-C-A and is now used to split the valyl-$tRNA^{Ile}$-C-C-A linkage. This mechanism very much resembles hydrolysis of peptides by trypsin. In this enzyme a serine is activated by an aspartic acid residue via the imidazole group of histidine. In our case the role of the serine is taken by the 3′-hydroxyl group of the substrate $tRNA^{Ile}$-C-C-A.

We would like to emphasize that, to the best of our knowledge, we see here the first case in which an enzyme with very high specificity does not fulfill its role by an extreme refinement of its original function. Instead it tolerates a mistake, forming a wrong product, which is finally corrected by a chemical proofreading rather than by a kinetic proofreading (Hopfield, 1974).

At the same time we can now give a rationale for our earlier discovery of the 2′ or 3′ specificity of the aminoacylation (Sprinzl et al., 1973). The two hydroxyl groups of the terminal ribose in tRNA have entirely different, even opposed functions: one is an accepting site, while the other has hydrolytic function.

By the subtle interplay between these two functions, a new type of enzymatic specificity has arisen in evolution.

Acknowledgment

We are very much indebted to Drs. H. Sternbach and H. Faulhammer for a gift of tRNA-nucleotidyl transferase and of tyrosyl-tRNA synthetase. The expert technical assistance of W. Hanewacker and J. Schischkoff during preparation of tRNAs and synthetases is gratefully acknowledged. We very much enjoyed many fruitful discussions with several colleagues, especially with Drs. D. Gauss, M. Sprinzl, G. Igloi, F. Seela, and J. Hobbs during the course of the work.

References

Baksht, E., deGroot, N., Sprinzl, M., and Cramer, F. (1975), *FEBS Lett. 55,* 105–108.

Baldwin, A. N., and Berg, P. (1966), *J. Biol. Chem. 241,* 839–845.

Beikirch, H., von der Haar, F., and Cramer, F. (1972), *Eur. J. Biochem. 26,* 182–190.

Bergmann, F. H., Berg, P., and Dieckmann, M. (1961), *J. Biol. Chem. 236,* 1735–1740.

Bonnet, J., and Ebel, J. P. (1972), *Eur. J. Biochem. 31,* 335–344.

Cramer, F., Faulhammer, H., von der Haar, F., Sprinzl, M., and Sternbach, H. (1975), *FEBS Lett. 56,* 212–214.

Eldred, E. W., and Schimmel, P. R. (1972), *J. Biol. Chem. 247,* 2961–2964.

Engel, G., Heider, H., Maelicke, M., von der Haar, F., and Cramer, F. (1972), *Eur. J. Biochem. 29,* 257–262.

Fasiolo, F., Remy, P., Pouyet, J., and Ebel, J. P. (1974), *Eur. J. Biochem. 50,* 227–236.

Faulhammer, H., and Cramer, F. (1974), FEBS Meeting, Budapest, Abstract f 10b40, p 475.

Fraser, T. H., and Rich, A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2671–2675.

Griffin, B. E., Jarman, M., Reese, C. B., Sulston, J. E., and Trentham, D. R. (1966), *Biochemistry 5,* 3638–3649.

Heider, H., Gottschalk, E., and Cramer, F. (1971), *Eur. J. Biochem. 20,* 144–152.

Hopfield, J. J. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4135–4139.

Kisselev, L. L., and Favorova, O. O. (1974), *Adv. Enzymol. 40,* 141–238.

Lagerkvist, U., Rymo, L., and Waldenstroem, J. (1966), *J. Biol. Chem. 241,* 5391–5400.

Loftfield, R. B. (1972), *Prog. Nucleic Acid Res. Mol. Biol. 12,* 87–128.

Loftfield, R. B., and Eigner, E. A. (1965), *J. Biol. Chem. 240,* 1482–1484.

McLaughlin, C. S., and Ingram, V. M. (1965), *Biochemistry 4,* 1442–1447.

Maelicke, A., Sprinzl, M., von der Haar, F., Khwaja, T. A., and Cramer, F. (1974), *Eur. J. Biochem. 43,* 617–625.

Pingoud, A., Riesner, D., Boehme, D., and Maass, G. (1973), *FEBS Lett. 30,* 1–5.

Prusiner, P., Brennan, T., and Sundaralingam, M. (1973), *Biochemistry 12,* 1196–1202.

Rymo, L., Lundvik, L., and Lagerkvist, U. (1972), *J. Biol. Chem. 247,* 3888–3897.

Schlimme, E., von der Haar, F., and Cramer, F. (1969), *Z. Naturforsch. B 24,* 631–637.

Schneider, D., Solfert, R., and von der Haar, F. (1972), *Hoppe-Seyler's Z. Physiol. Chem. 353,* 1330–1336.

Schreier, A. A., and Schimmel, P. R. (1972), *Biochemistry 11,* 1582–1589.

Shiloach, J., Lapidot, Y., deGroot, N., Sprinzl, M., and Cramer, F. (1975), *FEBS Lett. 57,* 130–133.

Söll, D., and Schimmel, P. R. (1974), *Enzymes, 3rd Ed. 10,* 489–538.

Sourgoutchov, A., Blanquet, S., Fayat, G., and Waller, J. P. (1974), *Eur. J. Biochem. 46,* 431–438.

Sprinzl, M., Scheit, K. H., Sternbach, H., von der Haar, F., and Cramer, F. (1973), *Biochem. Biophys. Res. Commun. 51,* 881–887.

von der Haar, F. (1973), *Eur. J. Biochem. 34,* 84–90.

von der Haar, F., and Cramer, F. (1975), *FEBS Lett. 56,* 215–217.

Wolfenden, R., Rammler, D. H., and Lipmann, F. (1964), *Biochemistry 3,* 329–338.

Yaniv, M., and Gros, F. (1969), *J. Mol. Biol. 44,* 17–30.