Editing Mechanisms in Aminoacylation of tRNA: ATP Consumption and the Binding of Aminoacyl-tRNA by Elongation Factor Tu†

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ABSTRACT: The stoichiometry of the hydrolysis of ATP and the aminoacylation of tRNA has been examined in order to determine whether the editing mechanism that is used to correct errors in the recognition of the amino acid also leads to the wasteful hydrolysis of ATP in the biologically correct reactions. The inhibition of the isoleucyl-tRNA synthetase catalyzed deacylation of Val-tRNA^{Ile} by binding to the elongation factor Tu–GTP complex has been examined to see if this could prevent editing. A systematic survey of nine aminocyl-tRNA synthetases, including the isoleucyl-tRNA synthetase under a wide range of conditions, shows that at least 0.95 mol of aminoacyl-tRNA is formed for every mol of ATP hydrolyzed. This is considerably higher than the stoichiometry of 0.66 ± 0.2 recently found for the isoleucyl-tRNA synthetase (Hopfield, J. J., Yamane, T., Yue, V., and Coutts, S. M. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1164). Reasons are suggested for the discrepancy between the two results. The only major waste of ATP hydrolysis appears to occur as a direct result of the activation of valine by the isoleucyl-tRNA synthetase followed by editing. This is calculated to occur in vivo with a frequency of 1 mol of valine activated per 16 mol of isoleucine. The binding of Val-tRNA^{Ile} by elongation factor Tu–GTP is found to decrease the rate of the isoleucyl-tRNA synthetase catalyzed deacylation by a factor of about 10^4. The turnover number for the hydrolysis, about 0.01 s^{-1}, is 100 times slower than the rate of turnover of aminoacyl-tRNA during protein synthesis. Thus, if there is an excess of elongation factor Tu in the cell, the amino acid from any misacylated tRNA released from the aminoacyl-tRNA synthetase will be incorporated into proteins. The current status of various postulated editing mechanisms is discussed in light of the above results.

Errors in protein synthesis caused by the misrecognition of amino acids by aminoacyl-tRNA synthetases are substantially reduced by *editing.* The editing mechanism is required when there are amino acids of the same size as or slightly smaller than the cognate amino acid that can also bind to the active site of the enzyme: for example, valine competing with isoleucine for the active site of the isoleucyl-tRNA synthetase and threonine competing with valine for the valyl-tRNA synthetase. The noncognate amino acids undergo the first step in the charging reaction, the formation of the enzyme-bound aminoacyl adenylate complex. However, on addition of tRNA to the complex, it hydrolyzes without the overall formation of aminoacyl-tRNA (Baldwin and Berg, 1966; Fersht and Kaethner, 1976b). The mechanism for the editing process with the valyl-tRNA synthetase and threonine involves the transfer of threonine to tRNA^{Val} followed by the rapid enzyme-catalyzed deacylation of the Thr-tRNA^{Val} before most of it leaves the enzyme (Fersht and Kaethner, 1976b). The editing mechanism for the rejection of valine by the isoleucyl-tRNA synthetase has many similar kinetic characteristics to this, but the transiently misacylated Val-tRNA^{Ile} cannot be trapped by rapid quenching methods in the same way as was Thr-tRNA^{Val} (Fersht, 1977a). It is possible that the rejection of valine by the isoleucyl-tRNA synthetase occurs in a two-step process with most of the valyl adenylate being destroyed before the transfer of valine to tRNA^{Ile}.

Whatever its detailed chemical mechanism, editing requires the expenditure of energy as 1 mol of ATP is hydrolyzed for the rejection of 1 mol of noncognate amino acid. A further question concerns whether the editing mechanism is so efficient that some of the biologically correct aminoacyl adenylate is destroyed in the successful charging of tRNA. In other words, does the formation of 1 mol of aminoacyl-tRNA require the hydrolysis of more than 1 mol of ATP? It is well documented that the addition of tRNA to isolated enzyme–aminoacyl adenylate complexes generally leads to incomplete transfer of the amino acid to the tRNA (Baldwin and Berg, 1966; Loftfield, 1972). Although it was suggested that this incomplete transfer could be related to the specificity mechanism, it was also noted that the addition of ATP to a reaction mixture containing tyrosyl-tRNA synthetase, tyrosine, tRNA, and inorganic pyrophosphatase (to drive the reaction to completion) led to the formation of at least 0.97 mol of Tyr-tRNA^{Tyr} for every mol of ATP hydrolyzed (Fersht and Jakes, 1975). The addition of ATP to the otherwise complete aminoacylation mixture is obviously a better model for the aminoacylation reaction in vivo than is the addition of tRNA to the preformed and isolated aminoacyl adenylate complex. Subsequent to this, Hopfield et al. (1976) have reported that the addition of ATP to a mixture of isoleucyl-tRNA synthetase, isoleucine, tRNA, inorganic pyrophosphatase, and the elongation factor Tu (the factor inhibits the enzymatic hydrolysis of the Ile-tRNA (Eldred and Schimmel, 1972)) leads to the formation of only 0.66 ± 0.2 mol of Ile-tRNA for every mol of ATP hydrolyzed, a value significantly less than one. However, continuing studies in our laboratory indicate that the value of close to 100% efficiency found for the synthesis of tyrosyl-tRNA is typical. In this study we present the results of a survey of nine representative enzymes and suggest a reason for the discrepancy between the two sets of results. We also examine the inhibition by Tu factor of the enzyme-catalyzed deacylation of mischarged tRNAs. Some proposed mechanisms for specificity are analyzed in light of these results.

Materials and Methods

Preparations of enzymes and tRNA have been described in previous papers from this laboratory (Fersht and Jakes, 1975; Fersht and Kaethner, 1976a,b; Mulvey and Fersht, 1976; Fersht, 1977a). ^{14}C-labeled amino acids were obtained from

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The Radiochemical Centre, Amersham, England. Unlabeled amino acids were obtained from BDH or Sigma and further purified by recrystallization from ethanol–water or water. ATP (disodium salt) was Sigma Grade and had a stated purity of 99%. Purified Tu factor was a generous gift from Dr. Reuben Leberman.

Solutions were dispensed from Hamilton syringes that had been calibrated by weighing or radioactive procedures. Concentrations of ATP were determined spectrophotometrically using either an extinction coefficient of 14.7 mM^{-1} cm^{-1} at 257 nm and pH 2 (PL Biochemicals Inc. Circular OR-10) or 14.9 mM^{-1} cm^{-1} at 260 nm in distilled water. The absorbance measurements were made using a Gilford spectrophotometer and checked using a Cary 17 spectrophotometer. Radioactivity was measured using a toluene-based scintillant (12.5 g of 2,5-diphenyloxazole and 0.75 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 2.5 L of toluene) and a Beckman LS-250 counter at an open window setting. Aqueous radioactive solutions were dried on Schleicher and Schüll BA 85 or Millipore nitrocellulose filters and suspended in the scintillant.

Measurements of rates and yields were made at 25.0 ± 0.1 °C in standard buffered solutions containing 10 mM $MgCl_2$, 0.1 mM mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride, and either Tris[1]-Cl (pH 7.78, 144 mM), or, where specifically noted, Bistris-Cl (pH 5.87, 13 mM).

Assay for Tu Factor. The Tu–GTP complex which binds aminoacyl-tRNA was prepared by incubating Tu factor (~10 μM), GTP (200 μM), phosphoenolpyruvate (5 mM), and pyruvate kinase (0.01 mg/mL = 2 units/mL) in the standard pH 7.78 buffer at 37 °C for 20 min. The solution was then stored on ice for short periods or in liquid nitrogen for longer. The concentration of the Tu–GTP complex was measured by its ability to inhibit the isoleucyl-tRNA synthetase catalyzed hydrolysis of Ile-$tRNA^{Ile}$. To a solution containing $tRNA^{Ile}$ (4.7 μM), [^{14}C]Ile (35 μM), ATP (5.7 μM), inorganic pyrophosphatase (5 units/mL), and Tu factor (about 0.5–1 μM) in the standard pH 7.78 buffer was added a solution of isoleucyl-tRNA synthetase to give a concentration of 4.2 μM. Aliquots were quenched with trichloroacetic acid after 10, 15, 20, and 25 min to assay the concentration of [^{14}C]Ile-tRNA. (Under these conditions in the absence of Tu all the Ile-tRNA is hydrolyzed in about 7 min as $t_{1/2} \sim 40$ s (Fersht, 1977a)). Extrapolation of the concentration of [^{14}C]Ile-tRNA back to zero time gives the concentration of Tu factor.

Efficiency of Assay for Charged tRNA. Partially purified $tRNA^{Tyr}$ (tyrosyl acceptance = 384 pmol/A_{260}) was preparatively aminoacylated (Jakes and Fersht, 1975) and carefully freed from excess [^{14}C]Tyr (37 mCi/mmol) by repeated gel filtration at pH 5.0 and 4 °C. A sample containing 1.67 A_{260} units (= 534 pmol of [^{14}C]Tyr-tRNA) was dried on a nitrocellulose filter and the radioactivity measured by scintillation counting (34 000 cpm). An identical sample was added to a 5% solution of trichloroacetic acid, the precipitate collected on a nitrocellulose filter and, after washing and drying, the radioactivity measured as before (33 490 cpm). The procedure was repeated but a further 3.1 A_{260} units of uncharged tRNA added before the acid precipitation (32 000 cpm).

Stoichiometry of Aminoacyl-tRNA Formation. Stock solutions were prepared containing aminoacyl-tRNA synthetase, cognate tRNA, cognate ^{14}C-labeled amino acid, and inorganic pyrophosphatase in the standard pH 7.78 or pH 5.87 buffer. The aminoacylation reaction was initiated by adding a solution of ATP (usually 30 μL) to a 100- or 200-μL sample of the reaction mixture. Aliquots of 10 or 20 μL were taken periodically and quenched with a 5% solution of trichloroacetic acid containing the unlabeled amino acid, and the radioactivity was assayed as described previously. The specific activity of the ^{14}C-labeled amino acid was measured by drying a sample of the amino acid solution on a nitrocellulose filter using the same volume and syringe as used for sampling the aliquots from the aminoacylation mixture.

In some experiments, Tu–GTP complex was included in the initial stock solution.

Isoleucyl-tRNA Synthetase Catalyzed Hydrolysis of Tu-Bound Val-$tRNA^{Ile}$. [^{14}C]Val-$tRNA^{Ile}$ was prepared by the procedure of Fersht (1977a) using fractionated $tRNA^{Ile}$ (isoleucine acceptance = 1100 pmol/A_{260}) to give a product containing 1060 pmol of [^{14}C]Val per A_{260} unit of $tRNA^{Ile}$. The rate of deacylation was measured by assaying aliquots from solutions containing Tu factor (1.5 or 3 μM), [^{14}C]Val-$tRNA^{Ile}$ (0.3–0.6 μM), isoleucyl-tRNA synthetase (0.1–3 μM), and various combinations of other ligands as later indicated in the standard pH 7.78 buffer.

Results

A. *Stoichiometry of Aminoacyl-tRNA Formation.* The accurate determination of the amount of tRNA charged during a reaction is complicated by two factors: (a) the precision of the assay; (b) the instability of the charged tRNA due to its enzyme-catalyzed hydrolysis.

(a) The assay procedure used in this study appears to be reliable and accurate. Precipitation of 1–2 A_{260} units of ^{14}C-labeled aminoacylated tRNA by trichloroacetic acid and collection of the precipitate on a Millipore or Schleicher and Schull nitrocellulose filter followed by drying and scintillation counting gives 98.5% of the number of counts found on drying a solution of the same sample on the filter. At higher concentrations of tRNA, the efficiency of the assay drops. On using sufficient quantities of tRNA to give a heavy precipitate and collecting on glass fiber filters, the efficiency drops by 10 to 20%. Thus, care was taken to use, as far as possible, relatively low concentrations of tRNA.

(b) The depletion of aminoacyl-tRNA by the enzyme-catalyzed hydrolysis becomes a serious problem when the hydrolytic rate approaches within a factor of ten of the rate of aminoacylation. Conversely, when the aminoacylation rate is relatively rapid and the hydrolysis slow, the time course for the concentration of charged tRNA may be easily and accurately extrapolated back to zero time. When this does not occur, the hydrolysis rate may be decreased by a number of procedures. (1) Increasing the tRNA concentration decreases the enzyme-catalyzed hydrolysis rate since uncharged tRNA is a competitive inhibitor of the binding of charged tRNA to the enzyme (Eldred and Schimmel, 1972). (2) Lowering the pH to about pH 6 greatly decreases the hydrolysis rate (Eldred and Schimmel, 1972). (3) The addition of Tu factor stabilizes the aminoacylated tRNA (Eldred and Schimmel, 1972; Hopfield et al., 1976). Of these, the last is the most satisfactory since it mimics conditions in vivo.

Examples of this behavior are given in Figures 1 and 2. In Figure 1 it is seen that tyrosyl-, valyl-, and methionyl-tRNA synthetases from *E. coli* rapidly aminoacylate their cognate tRNAs. The aminoacyl-tRNAs then hydrolyze according to precise first-order kinetics, the rate constant for hydrolysis of each being essentially independent of the concentration of added ATP. The tyrosyl-tRNA synthetase from *B. stearothermophilus* charges its tRNA relatively slowly under the reaction conditions. However, the formation and decay of the

[1] Abbreviations used: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; P_i, inorganic phosphate.

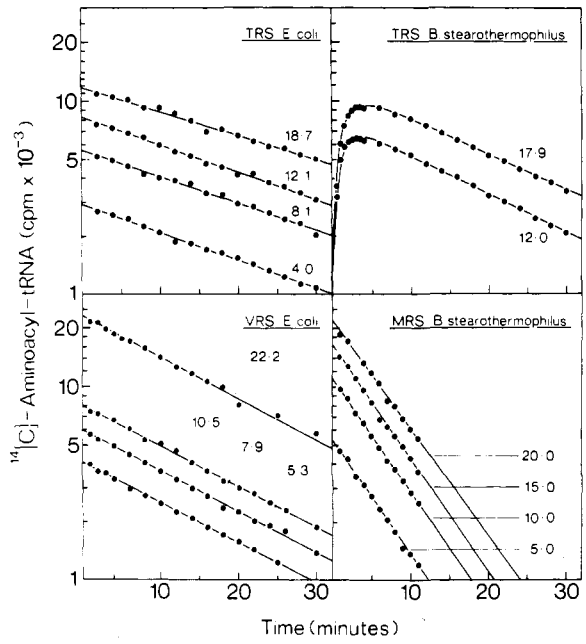


FIGURE 1: Time courses for the aminoacylation of tRNA by four enzymes at pH 7.78 and 25 °C. Initial concentrations of ATP (μM) are as in figure, other details are in Table I (TRS = tyrosyl-, VRS = valyl-, and MRS = methionyl-tRNA synthetase).

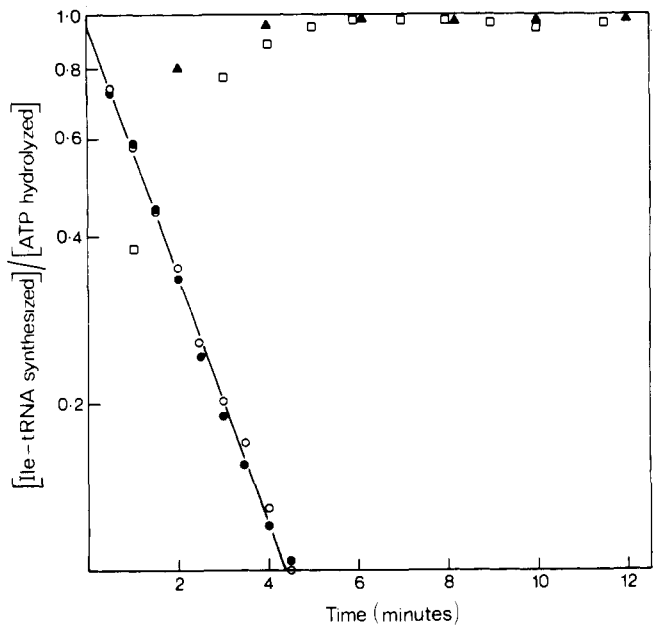


FIGURE 2: Time courses for the aminoacylation of tRNA by the isoleucyl-tRNA synthetase at 25 °C in the presence of inorganic pyrophosphatase. (I) With 2.7 μM enzyme, 14.4 μM tRNA^{Ile} (unfractionated), 110 μM isoleucine, pH 7.78 and (a) 5.24 μM (○), (b) 7.34 μM (●) initial concentration of ATP. (2) 0.96 μM enzyme, 3.5 μM tRNA (acceptance = 370 pmol of isoleucine/A_{260}), 38 μM isoleucine (pH 7.78), and an initial concentration of 2.29 μM ATP in the presence of 5.8 μM Tu-GTP complex (▲). (3) 3.6 μM enzyme, 34 μM tRNA^{Ile} (370 pmol/A_{260}), 100 μM isoleucine, pH 5.9, and an initial concentration of 19 μM ATP (□).

tyrosyl-tRNA nicely fit a theoretical curve for two consecutive first-order processes. The justification for the theoretical plot will be seen below. Figure 2, showing the reactions of the isoleucyl-tRNA synthetase, illustrates the various methods of slowing down the deacylation rate. At pH 7.78 the hydrolysis is very rapid (○ and ●). On lowering the pH to 5.87 and using a high ratio of tRNA to enzyme (□), the hydrolysis rate decreases sufficiently compared with the charging rate that the observed concentration of [^{14}C]Ile-tRNA plateaus at about 96% of the concentration of ATP added. Similarly, using Tu factor at pH 7.78 (▲) the [^{14}C]Ile-tRNA concentration reaches about 95–100% of the added ATP.

Time Course of [AA-tRNA]. The reasons for the simple exponential rise and decay of AA-tRNA during the experiments may be seen from the following analysis. At a fixed concentration of amino acid and variable concentrations of ATP, the rate of formation of aminoacyl-tRNA is found experimentally to be given by:

$$\frac{d[AA\text{-}tRNA]}{dt} = \frac{k_{cat}[E_0][ATP]}{K_M + [ATP]} \quad (1)$$

When $[ATP] \ll K_M$, as in the experiments, this reduces to

$$\frac{d[AA\text{-}tRNA]}{dt} = (k_{cat}/K_M)[E_0][ATP] \quad (2)$$

$$= k_1[ATP] \quad (3)$$

It may be shown by simple steady-state kinetics, assuming rapid binding of tRNA, that the enzyme-catalyzed deacylation rate (which is much faster than the water or hydroxide ion catalyzed route at pH 7.78) is given by:

$$\frac{-d[AA\text{-}tRNA]}{dt} = \frac{(k_h/K_S)[E_0][AA\text{-}tRNA]}{1 + [tRNA]/K_S' + [AA\text{-}tRNA]/K_S} \quad (4)$$

(where k_h is the turnover number for the reaction, $[E_0]$ the total concentration of enzyme, and K_S and K_S' are the dissociation constants of the AA-tRNA- and tRNA-enzyme complexes). When $[tRNA]/K_S' \gg [AA\text{-}tRNA]/K_S$, or, as has often been found experimentally, $K_S = K_S'$ (Yaniv and Gros, 1969; Hélène et al., 1971; Yarus and Berg, 1967; Eldred and Schimmel, 1972; Chousterman and Chapeville, 1973; Bartmann et al., 1974; Rouget and Chapeville, 1971), eq 4 reduces to:

$$\frac{-d[AA\text{-}tRNA]}{dt} = k_2[AA\text{-}tRNA] \quad (5)$$

The reaction scheme thus simplifies to:

$$ATP \xrightarrow{k_1} AA\text{-}tRNA \xrightarrow{k_2} AA + tRNA \quad (6)$$

The analytical solution for this is given by:

$$[AA\text{-}tRNA] = \frac{\alpha k_1[ATP_0]}{k_1 - k_2}[e^{-k_2 t} - e^{-k_1 t}] \quad (7)$$

(where α = number of moles of AA-tRNA formed per mole of ATP hydrolyzed).

Equations 6 and 7 are an approximation because the enzyme is in a different state during the early stage of the reaction when ATP is present. However, as all ligated forms of the aminoacyl-tRNA synthetases are hydrolytically active (Eldred and Schimmel, 1972; Yarus, 1972; Fersht and Kaethner, 1976b; Fersht, 1977a), they are good approximations. When $k_1 \gg k_2$, the approximations are excellent and the errors insignificant.

Experimental Stoichiometries. Experimental conditions, values of k_1 and k_2, and observed stoichiometries are given in Table I. In most cases k_1 is far greater than k_2 and is too fast to be seen. In these examples k_1 was calculated from steady-state measurements of k_{cat} and K_M for the aminoacylation reaction under similar conditions. The value of [AA-tRNA] found from extrapolation back to zero time was divided by a factor of $k_1/(k_1 - k_2)$ as indicated by eq 7. This factor is in all these cases very close to 1. In two examples, the phenylal-

TABLE I: Stoichiometry of tRNA Charging and ATP Hydrolysis.[a]

Aminoacyl-tRNA synthetase (concn, μM)	Cognate tRNA (μM)[b]	ATP (μM)[c]	k_1[d] (s^{-1})	k_2[e] (s^{-1})	Mol of tRNA charged/mol of ATP hydrolyzed
Isoleucyl- (*E.c.*)					
(2.73)	14 (50)	3-10 (4)	0.11	9×10^{-3}	0.93 ± 0.05
(2.8)	31 (370)	12-19 (2)	0.045	2.5×10^{-3}	0.90 ± 0.05
(3.62, pH 7.2)	36 (370)	5-19 (3)	0.016	2×10^{-3}	0.98 ± 0.02
(3.6, pH 5.9)	34 (370)	10-19 (4)	0.01	9×10^{-5}	0.99 ± 0.02
(1.5, pH 5.9)	9 (100)	1.5-2 (2)	0.005	3×10^{-4}	0.91 ± 0.05
Tyrosyl- (*E.c.*) (2.6)	36 (300)	4-21 (4)	0.03	5×10^{-4}	0.94 ± 0.03
Tyrosyl- (*B.s.*) (3)	37 (1400)	12-18 (2)	0.015	7×10^{-4}	0.97 ± 0.03
Valyl- (*E.c.*) (2.4)	46 (480)	5-22 (4)	0.13	8×10^{-4}	0.95 ± 0.02
Valyl- (*B.s.*) (3.2, pH 5.9)	32 (16)	8-16 (4)		0	0.96 ± 0.02
Methionyl- (*E.c.*) (2)	128 (900)	5-22 (4)	0.2	1.6×10^{-3}	1.00 ± 0.02
Methionyl- (*B.s.*) (2.3)	38 (160)	5-20 (4)	0.14	2.2×10^{-3}	0.99 ± 0.02
Arginyl- (yeast) (2.5)	18 (1440)	4-9 (3)	0.06	9.5×10^{-4}	1.01 ± 0.02
Phe- (yeast) (1.3)[f]	44 (1200)	10-40 (4)	0.026	1.6×10^{-3}	0.96 ± 0.05

[a] pH 7.78 and 25 °C unless otherwise stated. *E.c.* = *E. coli*; *B.s.* = *B. stearothermophilus*. [b] Amino acid acceptance in parentheses in units of pmol/A_{260} of total tRNA. [c] Number of experiments in parentheses. [d] First-order rate constant for the aminoacylation of tRNA. [e] First-order rate constant for the hydrolysis of aminoacyl-tRNA. [f] Fasiolo and Fersht (1977).

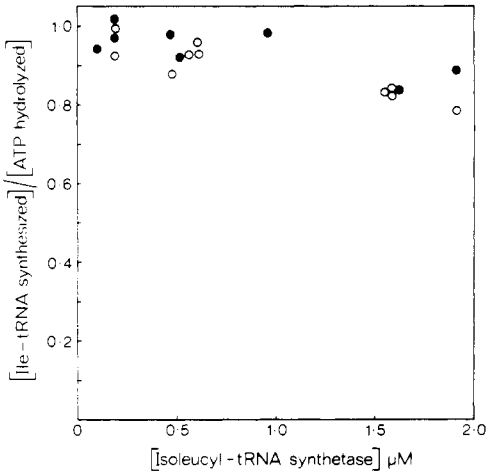


FIGURE 3: Stoichiometry of formation of Ile-tRNA^{Ile} from ATP at 25 °C, pH 7.78, in the presence of Tu-GTP (5.8 μM). (●) Fractionated tRNA (acceptance = 370 pmol of isoleucine/A_{260}); (○) unfractionated tRNA.

anyl-tRNA synthetase from yeast (Fasiolo and Fersht, 1977) and the tyrosyl-tRNA synthetase from *B. stearothermophilus*, the charging rates are sufficiently slow that the data were fitted directly to eq 7 (see Figure 1). Apart from some experiments with the isoleucyl-trna synthetase, it is seen in the last column of Table I that in every case at least 0.94 mol of AA-tRNA are formed for every mol of ATP hydrolyzed. The experiments with the isoleucine enzyme in the absence of Tu are the most difficult to analyze since k_2 is generally fast and appreciable compared with k_1. In the experiment at pH 5.9 and high concentration of tRNA where the hydrolysis rate is slow, a stoichiometry in the range 0.94 to 1.0 is observed. In the other experiments, where the errors are generally higher, values of between 0.9 and 1.0 are found. Because of this, we turned to the use of Tu factor to stabilize the isoleucyl-tRNA as described by Hopfield et al. (1976). The results are summarized in Figure 3. Using tRNA enriched for tRNA^{Ile} and enzyme concentrations below 1 μM, an average stoichiometry of 0.965 ± 0.014 was obtained. Under similar conditions, using unfractionated tRNA, a stoichiometry of 0.94 ± 0.02 is found. However, at higher enzyme concentrations the stoichiometry falls somewhat. Also, under these conditions using the unfractionated tRNA, the Tu factor does not completely inhibit the deacylation of the isoleucyl-tRNA and the measured concentrations were extrapolated back to zero time to calculate the total amount formed. The concentration of isoleucyl-tRNA synthetase in vivo is about 0.5 μM (Yarus and Berg, 1969), in the region where the higher stoichiometries are found.

Isoleucyl-tRNA Synthetase Catalyzed Hydrolysis of Tu-Bound Val-tRNA^{Ile}. The isoleucyl-tRNA synthetase catalyzed hydrolysis of Val-tRNA^{Ile} is not completely inhibited by the binding of the misacylated tRNA to the Tu-GTP complex (Table II and Figure 4). The activity is low with a turnover number of about 0.01 s^{-1}. The rate varies with enzyme concentration; the preliminary data indicate a K_M of about 0.5 μM. The addition of isoleucine, ATP, and inorganic pyrophosphatase to the reaction mixture to convert the enzyme to the aminoacyl adenylate complex does not decrease the activity and may cause a slight increase. The turnover number in the absence of Tu is 10 s^{-1} for the hydrolysis catalyzed by the free enzyme and 3.3 s^{-1} when catalyzed by the enzyme-isoleucyl adenylate complex. Binding to Tu therefore decreases the hydrolysis rate constant by a factor of about 10^4.

The low deacylation rate constant is important for two reasons. (a) The rate constant is, as discussed later, too low to allow the deacylation of any Val-tRNA:Tu-GTP formed in vivo. (b) The rate is sufficiently high to deacylate Val-tRNA:Tu-GTP formed in vitro in the experiments designed to trap misacylated tRNA. For example, using the kinetic data for the ATP:pyrophosphatase activity of the isoleucyl-tRNA synthetase (Fersht, 1977a) and the suggestion by Hopfield et al. (1976) that one molecule in 270 of valyl adenylate formed in the reaction successfully charges tRNA, it is calculated that, in the presence of 1 μM isoleucyl-tRNA synthetase, 26 μM valine, saturating concentration of ATP, and 0.33 μM tRNA^{Ile}, the complex Val-tRNA:Tu-GTP is formed at about 2×10^{-10} mol^{-1} L^{-1} s^{-1}. However, it is seen in Table II that the complex is deacylated at a rate of 0.03 [Val-tRNA:Tu-GTP] s^{-1}. Thus, there should not be a continuous accumulation of the mischarged tRNA but its concentration will reach a steady-state level of about 7 nM.

Discussion

The stoichiometry of the number of moles of aminoacyl-tRNA formed per mole of ATP hydrolyzed during the aminoacylation of tRNA was determined for a wide range of en-

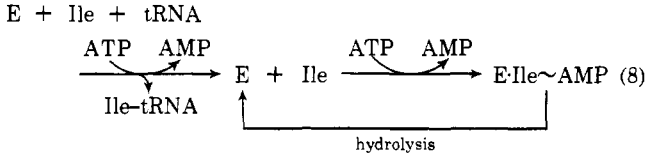
TABLE II: Isoleucyl-tRNA Synthetase-Catalyzed Deacylation of Val-tRNA^{Ile} Bound to Tu-GTP.[a]

Isoleucyl-tRNA synthetase (μM)	[Val-tRNA^{Ile}]$_0$ (μM)	[Tu-GTP] (μM)	Initial rate of hydrolysis (nM s^{-1})
1.5	0.48	3.9	7
0.14	0.48	3.9	2
0.3	0.48	3.9	2.6
0.3 (+ Ile-AMP)	0.48	3.9	2.8
0.9	0.33	1.0	10
0.9 (+ Ile-AMP)	0.33	1.0	10
0.4	0.33	1.0	6

[a] pH 7.78 and 25 °C.

zymes and by a variety of methods to be in the range 0.95 to 1.00. The basic experimental procedure consisted of the addition of a known amount of ATP to an otherwise complete reaction mixture containing the ^{14}C-labeled amino acid followed by the measurement of the ^{14}C-labeled aminoacyl-tRNA formed on total hydrolysis of the ATP. The accuracy of the method depends on the determination of the concentration of the added ATP and the efficiency of the acid precipitation assay for charged tRNA. The concentration of ATP was determined spectrophotometrically assuming a purity of 99% as stated by the manufacturers. The efficiency of the assay for charged tRNA was found from calibration experiments to be 98.5% under optimal conditions. Systematic errors from using these numbers should not overestimate the stoichiometry by more than 2.5%. It is more likely that the systematic errors involved lead to underestimates of the stoichiometry: any hydrolysis of the ATP occurring during storage or in the reaction mixture will lower the apparent stoichiometry. Similarly, high concentrations of protein or tRNA lower the efficiency of the scintillation counting of the precipitated radioactive material. Thus, the stoichiometries may be even closer to 1.00 than indicated in Table I.

The most difficult stoichiometry to measure is that involving the isoleucyl-tRNA synthetase because of the high hydrolytic activity of the enzyme toward Ile-tRNA^{Ile}. However, using Tu-GTP to stabilize the aminoacylated tRNA as recommended by Hopfield et al. (1976), it is found that close to 0.95 mol of Ile-tRNA^{Ile} is formed per mol of ATP hydrolyzed. The stoichiometry *appears* to drop slightly at high enzyme concentrations but under these conditions the precipitation assay is less accurate and the enzyme concentrations are far higher than in vivo. The value of 0.95 is considerably greater than the stoichiometry of 0.66 ± 0.2 found by Hopfield et al. (1976). One possible reason for the discrepancy between the two sets of results is the large error quoted for the latter experiments. Hopfield et al. (1976) measured the simultaneous hydrolysis of ATP and charging of tRNA by using both [γ-^{32}P]ATP and labeled amino acid. Many manipulations are involved in separating the isotopes and determining the ATP consumption that could lead to large errors. However, an even more important reason for the discrepancy appears to come from an experimental artefact. Their procedure uses an addition of an excess of ATP over tRNA in the reaction mixture. Thus, ATP is first consumed in charging the tRNA and then further ATP is used in forming enzyme-bound isoleucyl adenylate (eq 8).



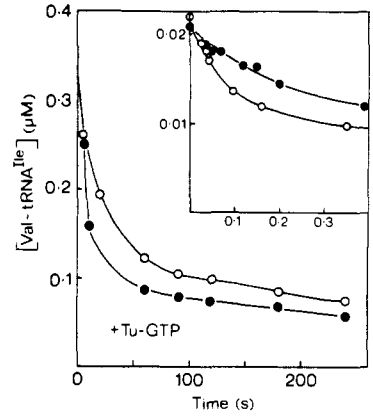


FIGURE 4: Inhibition by Tu-GTP of the isoleucyl-tRNA synthetase catalyzed deacylation of Val-tRNA^{Ile} at 25 °C and pH 7.78. Body of figure: 0.94 μM enzyme, 1 μM Tu-GTP (○), plus Ile-AMP (●). Inset: absence of Tu:GTP (data from Fersht, 1977a).

As their reaction mixtures contained about 0.16 μM isoleucyl-tRNA synthetase and 0.8 μM tRNA^{Ile}, the stoichiometry must be corrected by 20% to a value of 0.8 ± 0.2 or greater, a value not inconsistent with our results. Our experimental procedure uses an excess of tRNA over ATP so that this error is avoided.

Energy Expenditure in Editing. The enzyme which has the most difficult specificity problem and hence requires the greatest wasteful hydrolysis of ATP is the isoleucyl-tRNA synthetase. Valine binds only 100 times more weakly than isoleucine but is present at a five- to sixfold higher concentration in *E. coli* (Raunio and Rosenqvist, 1970). Using the relationship that the relative amounts of Val-AMP and Ile-AMP formed by the enzyme in a mixture of valine and isoleucine is given by $(k_{cat}/K_M)_{Val}[Val]/(k_{cat}/K_M)_{Ile}[Ile]$, where k_{cat}/K_M refers to the rate constants for the activation of amino acid in the presence of tRNA (Fersht, 1974, 1977b), it may be calculated from the data of Fersht (1977a) that 1 mol of valine is activated for every 16 mol of isoleucine. Therefore only 6% of the ATP consumed is wasted in activating the noncognate amino acid. The other aminoacyl-tRNA synthetases activate a smaller proportion of noncognate amino acids and waste less ATP. The valyl-tRNA synthetase from *E. coli* activates only a small amount of threonine since there is 40 times more valine than threonine (Raunio and Rosenqvist, 1970), and k_{cat}/K_M is 320 times higher for the activation of valine. Thus, only 1 mol of threonine is activated by the valyl-tRNA synthetase for every 1.3×10^4 mol of valine and only 0.01% of the ATP wastefully hydrolyzed because of incorrect activation. Thus, apart from the isoleucyl-tRNA synthetase, less than 5% of the ATP used in activating the amino acids is wastefully hydrolyzed because of editing since the stoichiometry of formation of the correctly aminoacylated tRNA is 0.95 or greater (Table I).

Implications for Editing Mechanisms. The demonstration by Baldwin and Berg (1966) that the addition of tRNA^{Ile} to the isoleucyl-tRNA synthetase:valyl adenylate complex causes its hydrolysis showed experimentally the occurrence of editing mechanisms. Since then, several suggestions have been made concerning their chemical mechanisms and their precise roles in correcting errors. The data in the present paper allow a distinction between some of these possibilities.

Hopfield "Kinetic Proofreading". A basic problem in specificity is the rejection by an enzyme of a substrate which is smaller than or isosteric with the biologically "correct" substrate since the smaller substrate must be able to bind in

TABLE III: Upper Limits of Dissociation Rate Constants of Cognate Enzyme:Aminoacyl Adenylate Complexes.[a]

Aminoacyl-tRNA synthetase (source)	Upper limit of dissociation rate constant (s^{-1})
Isoleucyl- (*E.c.*)	1.7×10^{-3}
(Isoleucyl + Val-AMP)	(4×10^{-2})
Valyl- (*E.c.*)	9×10^{-4}
Valyl- (*B.s.*)	9×10^{-4}
Tyrosyl- (*E.c.*)	3×10^{-3}
Tyrosyl- (*B.s.*)	4×10^{-4}
Methionyl- (*E.c.*)	2×10^{-3}
Methionyl- (*B.s.*)	3×10^{-3}

[a] The rate constant quoted is that for the rate of replacement of aminoacyl adenylate in the enzyme:AA-AMP complex by a newly formed molecule from excess ATP and AA, i.e.

$$\text{E:AA-AMP} \xrightarrow{\text{slow}} \text{E} + \text{AA} + \text{AMP}$$

$$\text{E} + \text{AA} + \text{AMP} \xrightarrow[\text{fast}]{\text{ATP, AMP}} \text{E:AA-AMP}$$

The rate of turnover is caused either by the prior (rate determining) dissociation of the complex or by the hydrolysis of the enzyme-bound complex if hydrolysis is faster than dissociation. The rate of turnover of complex is thus equal to or greater than the rate of dissociation of the complex. Turnover rates for the various complexes have been measured either by the rate of loss of label from E:[^{14}C]AA-AMP in the presence of saturating concentrations of AA and ATP (Fersht, 1975), or from the rate of consumption of ATP in active site titration experiments (Fersht et al., 1975). Data collected for pH 7.78 and 25 °C from published data from this laboratory.

the cavity at the active site constructed for the "correct" substrate (a larger competitive substrate may be excluded from a smaller active site by steric hindrance). The smaller substrate reacts more slowly as there is less potential binding energy available for catalysis and binding. The enzymic discrimination between the two substrates is proportional to the differences in their potential binding energies and cannot be increased beyond this by any mechanism such as "strain", induced fit, nonproductive binding, series of sequential reactions, interacting active sites, etc. (Fersht, 1974; and for detailed discussion, Fersht, 1977b). The only way to increase the enzymic discrimination between the substrates is by an editing mechanism where a covalent intermediate or product in the reaction is destroyed in a hydrolytic step (i.e., there is a branch point on the pathway through which the undesired product can be channelled). The simplest editing mechanism "kinetic proofreading", proposed by Hopfield (1974), is illustrated in its basic form in eq 9.

$$\begin{array}{ccccc} & \overset{\text{ATP}}{\curvearrowright}\ \overset{\text{PP}_i}{} & & & \\ \text{E·AA} & \longrightarrow & \text{E·AA-AMP} & \xrightarrow{k_3} & \text{AA-tRNA} \\ k_1 \uparrow\downarrow k_{-1} & & k_4 \uparrow\downarrow k_{-4} & & \\ \text{E} + \text{AA} & & \text{E} + \text{AA-AMP} & \xrightarrow{k_H} & \text{AA} + \text{AMP} \end{array} \qquad (9)$$

This utilizes the difference in association constants between the "correct" and "incorrect" substrates twice over by invoking the dissociation of the intermediate (k_4 and k_{-4}) as well as the initial complex (k_1 and k_{-1}). It is proposed that discrimination occurs via the two "off" rate constants k_{-1} and k_{-4}. For the "incorrect" substrate, $k_{-4} \gg k_3$ so that the aminoacyl adenylate diffuses into solution via k_{-4} where it rapidly hydrolyzes faster than the amino acid is transferred to tRNA via k_3. The fraction of aminoacyl adenylate that is successfully transferred is thus $k_3/(k_3 + k_{-4})$. The Hopfield mechanism has the limitation that, if the discrimination between the two substrates in the first step is f, the overall discrimination is f^2 or less. In order for the maximum discrimination to occur, k_{-4} must be comparable with k_3 for the *correct* substrate. For example, assuming for convenience as did Hopfield (1974) that k_3 is the same for both substrates as is k_1 and k_4, and denoting the incorrect substrate by ′, the discrimination at the second (editing) step is given by:

$$[k_3/(k_3 + k_{-4})]/[k_3/(k_3 + k_{-4}')] \equiv (k_3 + k_{-4}')/(k_3 + k_{-4}) \qquad (10)$$

Thus, for the discrimination to be at the maximum value of k_{-4}'/k_{-4}, k_3 must be less than both k_{-4}' *and* k_{-4}. Kinetic proofreading requires an appreciable waste in hydrolysis of ATP. The high stoichiometry of 0.95 or greater found in this study for the aminoacylation of tRNA^{Ile} is strong evidence against kinetic proofreading as formulated by Hopfield. If 95% of the isoleucyl adenylate is converted to tRNA^{Ile}, then $k_3/(k_3 + k_{-4}) = 0.95$ and so $k_3/k_{-4} = 20$. Now, since valine binds only 100 times more weakly than does isoleucine to the isoleucyl-tRNA synthetase (Fersht, 1977a), $k_{-4}' = 100\,k_{-4}$, and thus $k_{-4}' = 5k_3$. This means that one molecule in six of valyl adenylate that is formed should be transferred to tRNA^{Ile}. This is far too high, and considerably more than the value of one in 270 suggested experimentally by Hopfield et al. (1976). (Although the calculation is done specifically for the mechanism of eq 9, similar results will be obtained for any variation of the "kinetic proofreading" scheme). It should be noted that, even if the low stoichiometry of 0.66 found by Hopfield et al. (1976) were correct, it would not provide evidence for the "kinetic proofreading" mechanism since any postulated editing mechanism is likely to cause some wasteful hydrolysis of ATP.

Other evidence against the "kinetic proofreading" model is as follows. (1) The mechanism for the rejection of threonine by the valyl-tRNA synthetase has been solved and shown directly to involve a different mechanism (Fersht and Kaethner, 1976b). (2) The kinetic proofreading scheme requires that a reactive intermediate diffuse rapidly into solution where it hydrolyzes nonenzymically. However, it is found that the addition of tRNA to the isoleucyl-tRNA synthetase:Val-AMP complex causes a far more rapid hydrolysis of Val-AMP than occurs in solution (Baldwin and Berg, 1966; Fersht, 1977a). (3) The dissociation rate constant of Val-AMP from the isoleucyl-tRNA synthetase has been measured and is far too slow to be included in the kinetic proofreading scheme (Fersht, 1977a). Similarly, it is seen from Table III that the dissociation rate constants of cognate enzyme:aminoacyl adenylate complexes are very slow in general.

Inhibition of the Enzyme-Catalyzed Deacylation of Mischarged tRNA by Tu Factor. The discovery that aminoacyl-tRNA synthetases may catalyze the rapid deacylation of mischarged tRNAs has led to the suggestion that errors in recognition of both amino acid and tRNA may be edited by this reaction. In eq 11, an aminoacyl-tRNA synthetase acylates its cognate tRNA with a noncognate amino acid, which is then removed by the same enzyme (Eldred and Schimmel, 1972). In eq 12, a noncognate tRNA is acylated with the cognate amino acid and the error is edited by a different enzyme, the one specific for the noncognate tRNA.

$$\text{AA}' \xrightarrow{E_{AA}} \text{AA}'\text{-tRNA}^{AA} \xrightarrow{E_{AA}} \text{AA}' + \text{tRNA}^{AA} \qquad (11)$$

$$\text{AA} \xrightarrow{E_{AA}} \text{AA-tRNA}^{AA'} \xrightarrow{E_{AA'}} \text{AA} + \text{tRNA}^{AA'} \qquad (12)$$

These mechanisms must be evaluated in the light of the observation that the hydrolysis of the Tu-bound tRNA is very

slow. It may be calculated from published data on the rates of protein elongation, concentrations of aminoacyl-tRNA and Tu in *E. coli* (Dennis and Bremer, 1974; Cassio and Mathieu, 1974; Forchhammer and Lindahl, 1971; Skjold et al., 1973; Furano, 1975) that most of the tRNA in *E. coli* is aminoacylated, bound to Tu factor, and each mole of aminoacyl-tRNA turns over at about 1 s^{-1}. The rate of incorporation of the amino acid from the Tu-bound aminoacyl-tRNA into proteins is therefore several orders of magnitude faster than its enzyme-catalyzed deacylation. The importance of enzyme-catalyzed deacylation of mischarged tRNA depends crucially on whether or not there is more (accessible) Tu than aminoacyl-tRNA in the cell. Unfortunately, the figures are not known with sufficient precision.

If there is an excess of (accessible) Tu over aminoacyl-tRNA in the cell, editing by the enzyme-catalyzed deacylation of mischarged tRNA must occur *before* the aminoacyl-tRNA leaves the aminoacyl-tRNA synthetase. The editing cannot be used for the correction of mischarging caused by the misrecognition of tRNA as in eq 12, since the mischarged tRNA has to be transferred to a second aminoacyl-tRNA synthetase and become free in solution. The editing can be used for removing errors caused by the misrecognition of the amino acid as in eq 11, provided that the enzyme-catalyzed deacylation of the mischarged tRNA is faster than the rate of dissociation of the enzyme:aminoacyl-tRNA complex. Indeed, in the one example where it has been shown that editing occurs through the enzyme-catalyzed deacylation of the mischarged tRNA, the rejection of threonine by the valyl-tRNA synthetase of *B. stearothermophilus*, it was pointed out that the deacylation occurred before the Thr-$tRNA^{Val}$ dissociated from the enzyme (Fersht and Kaethner, 1976b).

Even if there is an excess of aminoacyl-tRNA over accessible Tu in the cell, editing by the deacylation of mischarged tRNA must take place with k_{cat} far greater than the turnover number for the incorporation of the amino acid into proteins. Since 1 mol of amino acid per mol of tRNA per second is incorporated into proteins, a value of 1 s^{-1} or less for k_{cat} for deacylation causes insignificant editing and a value of 10 s^{-1} only reduces errors by a factor of 10. In general, deacylation rates are much lower than this (Bonnet and Ebel, 1974).

To conclude, efficient editing requires that either the mischarged tRNA is deacylated before it can leave the aminoacyl-tRNA synthetase, as found for the rejection of threonine by the valyl-tRNA synthetase, or the misformed aminoacyl adenylate is destroyed before transfer to the tRNA, as may possibly occur in the rejection of valine by the isoleucyl-tRNA synthetase.

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