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Direct experimental evidence for kinetic proofreading in amino acylation of $tRNA^{Ile}$

(stoichiometry of energy coupling/amino acyl tRNA synthetase/error rate in biosynthesis)

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ABSTRACT **Kinetic proofreading is a reaction scheme with a structure more complicated than that of Michaelis kinetics, which leads to a proofreading for errors in the recognition of a correct substrate by an enzyme. We have measured the stoichiometry between ATP hydrolysis and $tRNA^{Ile}$ charging, using the enzyme isoleucyl-tRNA synthetase [L-isoleucine:$tRNA^{Ile}$ ligase (AMP-forming), EC 6.1.1.5] and the amino acids isoleucine (correct) and valine (incorrect). The enzymatic deacylation of charged tRNA, which would normally prevent meaningful stoichiometry studies, was eliminated by the use of transfer factor Tu·GTP, (which binds strongly to charged tRNA) in the reaction mixture. For isoleucine, 1.5 ATP molecules are hydrolyzed per tRNA charged, but for valine, 270. These stoichiometry ratios are fundamental to kinetic proofreading, for the energy coupling is essential and proofreading is obtained only by departing from 1:1 stoichiometry between energy coupling and product formation. Within the known reaction pathway, these ratios demonstrate that kinetic proofreading induces a reduction in errors by a factor of 1/180. An overall error rate of about 10^{-4} for tRNA charging is obtained by a kinetic proofreading using a fundamental discrimination level of about 10^{-2}, and is compatible with the low *in vivo* error rate of protein synthesis.**

Many biochemical reactions, particularly those associated with protein synthesis or DNA replication, exhibit high specificity in the selection between similar substrates. The overall error rate in selecting between two similar amino acids (1) in protein synthesis is believed to be about 3 in 10^4. The normal error rate in DNA synthesis (without post-replication repair) is about 1 in 10^8 or 10^9. These low error rates are biologically essential.

The elementary description of specificity in biochemical reactions is based on discrimination in a Michaelis complex. Suppose enzyme c is to recognize substrate C, reacting on it to produce $C_{product}$, but should not recognize similar substrate D. Within an elementary kinetic framework

$$\begin{aligned} C + c &\rightleftarrows Cc \longrightarrow C_{product} + c \\ D + c &\rightleftarrows Dc \longrightarrow D_{product} + c \end{aligned} \qquad [1]$$

The relative amounts of $C_{product}$ and $D_{product}$ (if C and D are present in equal concentrations) are determined by the kinetic parameters for the two reactions. Since c is supposed to recognize C, we suppose that along the reaction pathway the free energy for C is generally lower than for D. This kind of elementary reaction has an ability to discriminate between C and D that is limited by the maximum depression ΔG of the free energy of C below D along the reaction path. The smallest fraction of errors obtainable in an elementary fashion is

$$f_0 = \exp(-\Delta G/RT). \qquad [2]$$

When C and D are sufficiently similar, ΔG will not be large, and f_0 may be smaller than would be biologically optimal.

"Kinetic proofreading" (2) is a method of using twice (or more) the same Michaelis kinetic ability to distinguish between C and D, resulting in an error rate as small as f_0^2 (or higher power) instead of f_0 for a given ΔG. The essential features of kinetic proofreading are contained in the reaction scheme

$$\begin{array}{ccccccc} C + c & \rightleftarrows & Cc & \xrightarrow{\text{energy}} & (Cc)^* & \longrightarrow & c + C_{product} \\ & & & & \downarrow & & \\ & & & & C' + c & & \end{array} \qquad [3]$$

The first reaction step reversibly forms the usual Michaelis complex. The second step is enzymatically coupled to an energy source, typically the hydrolysis of a nucleoside triphosphate, and is strongly enough driven to be essentially irreversible. $(Cc)^*$ is a high energy intermediate, which can decompose in *two* ways: to free enzyme plus product *or* to free enzyme and nonproduct C'. C' can be either the original substrate C or a chemically modified form thereof.

This scheme is capable of proofreading. For equal concentrations of C and D, the discrimination against D in the initial Michaelis complex and in the formation of $(Dc)^*$ will be f_0. If the kinetic constants are appropriate (and they can be made so without enhancing ΔG at all) $(Dc)^*$ can be made to decay yielding chiefly D', while $(Cc)^*$ will decay yielding chiefly $C_{product}$. That D which in error managed to get to $(Dc)^*$ is discriminated against a second time. A proofreading scheme identical in mathematical principle but somewhat different in reaction topology was independently proposed by Ninio (3).

We present here experiments to test for proofreading in the discrimination between valine and isoleucine during the charging of $tRNA^{Ile}$ by the enzyme isoleucyl- tRNA synthetase [L-isoleucine:$tRNA^{Ile}$ ligase (AMP-forming), EC 6.1.1.5] (denoted by E). The overall region for correct charging is

$$\text{ATP} + \text{Ile} + tRNA^{Ile} \xrightarrow{E} \text{AMP} + \text{P} \sim \text{P} + \text{Ile·}tRNA^{Ile}. \qquad [4]$$

The overall scheme for *mischarging* is the same, valine being substituted for isoleucine (but keeping the isoleucyl-tRNA synthetase and $tRNA^{Ile}$). The steps in the reaction scheme include (4, 5)

Abbreviations: E, isoleucyl-tRNA synthetase; Tu, transfer factor Tu.

$$\text{Ile} + E + \text{ATP} \rightleftharpoons \text{Ile-}E\text{-ATP} \xrightarrow{P \sim P} E\text{-(Ile-AMP)} + \text{tRNA} \rightleftharpoons \text{tRNA-}E\text{-(Ile-AMP)} \quad [5]$$

$$\text{AMP} + E + \text{tRNA} + \text{Ile} \leftarrow E\text{-(tRNA-Ile)} \rightleftharpoons E + \text{Ile-tRNA (product)}$$

While this scheme has more complicated details than the skeleton illustrated for kinetic proofreading, it contains the three essential points in correct order: an initial Michaelis complex Cc (Ile-E-ATP), an "irreversible" driving step involving phosphate hydrolysis, and a high energy intermediate E-(tRNA^{Ile}-Ile) capable of breaking up in *two* ways, yielding either $C_{product}$ (Ile-tRNA^{Ile}) or nonproduct C' (tRNA^{Ile} + Ile). The method (order) of formation of the initial Ile-E-ATP complex is not important in the present context.

A measure of how many times the energy coupling step

$$Cc \xrightarrow{\text{energy}} (Cc)^* \quad [6]$$

is used *per product molecule* formed tests the proofreading hypothesis. Within a Michaelis-type scheme, with no exit path to $C' + c$, there will be a 1:1 stoichiometry between the number of product molecules formed and the number of times the energy cycle was used, *regardless of substrate*. If proofreading is being used, the stoichiometry should not be 1:1. With proofreading, when D is being used as substrate, the energy cycle will be used many times per $D_{product}$ molecule formed, for most of the $(Dc)^*$ will become $D' + c$. But when C is used as substrate, a number only somewhat larger than one energy cycle per $C_{product}$ molecule should result, for most of the $(Cc)^*$ molecules will yield $C_{product}$. In the charging of tRNA^{Ile} we have therefore measured the stoichiometry between ATP hydrolysis and tRNA charging for correct (isoleucine) and for incorrect (valine) substrates.

MATERIALS AND METHODS

The isoleucyl-tRNA synthetase (E) from *Escherichia coli* B was isolated by a modification of the method developed by Baldwin and Berg (6), as described by Eldred and Schimmel (7), and stored at −20° in 50% (vol/vol) glycerol. Unfractionated tRNA from *E. coli* H was obtained from Schwarz BioResearch and had an isoleucine acceptor activity of approximately 25 pmol/A_{260} at neutral pH. This corresponds to 1.6% active tRNA^{Ile} in the crude extract.

Purified transfer factor Tu, prepared as described by Miller and Weissbach (8), was supplied by D. Miller at concentrations of 7.7 mg/ml and 43 mg/ml. Tu-GTP complex was formed in an incubation mixture (90 μl) containing 0.05 M Tris-HCl buffer (pH 8.0), 0.01 M $MgCl_2$, 6 mM phosphoenol-pyruvate, 10 μg of crystalline phosphoenolpyruvate kinase, 22.5 nmol of GTP, and 0.5 nmol of Tu-GDP. The mixture was incubated for 20 min at 37° and then chilled on ice.

The [γ-^{32}P]ATP was purchased in 1 mCi lots (6.92 Ci/mmol and 26.8 Ci/mmol) from New England Nuclear; L-[4,5-^{3}H]isoleucine (83.4 Ci/mmol), L-[^{14}C]isoleucine (283 mCi/mmol), and L-[^{3}H]valine (1.3 Ci/mmol) were also from New England Nuclear. Unlabeled ATP, GTP, L-valine, and L-isoleucine were products of Calbiochem. Inorganic pyrophosphatase (2700 units/mg) was obtained from Worthington and pyruvate kinase, from Calbiochem.

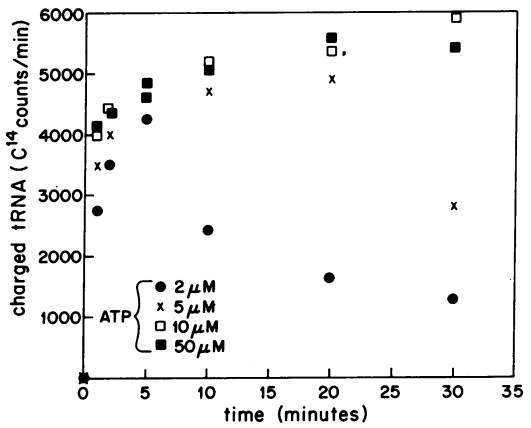

FIG. 1. The time dependence of the amount of tRNA charged with isoleucine as a function of ATP concentration. Initial conditions: [isoleucine] = 50 μM, [E] = 0.14 μM, [tRNA^{Ile}] ≈ 1 μM, pyrophosphatase 20 ng, [$MgCl_2$] = 15 mM, 50 mM Tris-HCl at pH 8. Total reaction volume = 0.1 ml. ATP levels were as indicated.

The aminoacylation reaction mixture was 50 μM in unfractionated tRNA (tRNA^{Ile} comprising about 2–3%, and active tRNA^{Ile} 1.6%, the concentration of active tRNA^{Ile} was 0.8 μM); 0.16 μM in E, 72 μM in L-isoleucine or L-valine and 15 μCi/100 μl of L-[^{3}H]isoleucine or L-[^{3}H]valine; various amounts of ATP from 5 μM to 100 μM, and 2 μCi/100 μl of [γ-^{32}P]ATP; 10 mM 2-mercaptoethanol, 20 ng/100 μl of inorganic pyrophosphatase, 0.5 nmol/100 μl of Tu-GTP complex, and 0.1 M $MgCl_2$. The mixture was buffered at pH 8.0 with 0.05 M Tris-HCl and was incubated at 37°. Samples of 50 μl were taken at certain time intervals; 25 μl were used for the determination of the amount of aminoacylation and 25 μl were used for ATP hydrolysis. Aminoacylation was detected by precipitation in 5% trichloroacetic acid; the precipitate was filtered through a Millipore membrane filter (HAWP, 25 mm), washed five times with 5 ml portions of cold 5% trichloroacetic acid and dried and counted in a liquid scintillation counter (Beckman LS-230). The amount of ATP hydrolyzed during the reaction was measured by detecting the quantity of $^{32}P_i$ released according to the procedure described by Modolell and Vazquez (9). A reversed-phase chromatography (RPC-5) column (1 × 25 cm) was prepared according to Pearson *et al.* (10).

RESULTS

When the charging reaction was carried out with isoleucine, limited available ATP, but *without* Tu-GTP, the amount of charged tRNA formed as a function of time was as shown in Fig. 1. At 2 μM ATP, the charged tRNA reaches a maximum at 5 min and falls off. At 5 μM ATP, a large decrease in the amount of charged tRNA occurs after 30 min. Higher ATP concentrations yield charging levels that are independent of the ATP level and plateau after about 15 min. The form of these curves is determined by the enzymatic deacylation reaction (11, 12)

$$\text{Ile-tRNA}^{Ile} \xrightarrow{E} \text{Ile} + \text{tRNA}^{Ile} \quad [7]$$

Under the present reaction condition (pH 7.8, 37°) the nonenzymatic deacylation amounts to about 15% of the total deacylation after 30 min (12–15). Whereas most of the tRNA became momentarily charged even at 2 μM ATP, a constant supply of new ATP and new charging reaction are needed to keep most of the tRNA molecules charged in competition with reaction 7. From the 2 and 5 μM charging curves, we have concluded that in the charging reaction itself (were it to take place without deacylation competition) fewer than two ATP molecules were required to charge one tRNA molecule, though it is difficult to quantitate this precisely without more detailed kinetic studies. The *recharging* necessitated by the enzymatic deacylation reaction is responsible for the consumption of almost all the ATP even at 5 μM concentrations. Thus in this circumstance, if sufficient (>5 μM) ATP is initially present, about 150 pmol of ATP will be consumed during the first 30 min. Of this quantity, only about 30 pmol will be necessary to charge $tRNA^{Ile}$ with isoleucine. The other 120 pmol will be consumed to recharge tRNAs which have been deacylated by the enzyme. This excess hydrolysis is in quantitative agreement with the V_{max} of about 0.7 min^{-1} determined (12) for the enzymatic deacylation under similar conditions.

The enzymatic deacylation reaction is *much* faster for the reaction (11)

$$\text{Val-tRNA}^{Ile} \xrightarrow{E} \text{Val} + \text{tRNA}^{Ile}. \qquad [8]$$

and will seriously interfere with the attempt to study fidelity of the forward reaction. Indeed, *E* had not been observed to charge $tRNA^{Ile}$ with valine at all. To eliminate the enzymatic deacylation [7, 8], Tu·GTP (from *E. coli*) was added to the reaction solution. This complex binds charged tRNA very tightly (16), and uncharged tRNA much less. Tu is an abundant protein in *E. coli* and the ratio Tu:tRNA is approximately 1:1 at all growth rates (17). In normal protein synthesis in *E. coli*, a charged tRNA is bound into a ternary complex (aa-tRNA)·Tu·GTP which is then, as a whole, bound to a ribosome (18). Because Tu recognizes aminoacylated tRNA, it is expected to protect charged tRNA from deacylation.

When Tu was added to a reaction volume in which isoleucine was the amino acid and ATP concentration was 1 mM, the amount of Ile·tRNA formed as a function of time was independent of the presence of Tu. This was expected to be the case, for with abundant ATP and a slow enzymatic deacylation, the tRNA could be kept essentially fully charged with isoleucine. The effect of Tu on the charging with valine was dramatic. Whereas without Tu, no charging with valine was detectable, with Tu, 1 mM ATP, and valine, the level of (mis)charging was typically 20% after 30 min. Thus, by using Tu·GTP as a "sink" for charged tRNA, the enzymatic deacylation [7, 8] can be chiefly eliminated, and the desired reaction can be studied without competition.

The stoichiometry experiments were carried out at ATP concentrations far lower than the usual millimolar concentration used for standard charging experiments. This was necessary to keep the background (chiefly due to radioactive P_i present in the ATP) from overwhelming the desired reaction. In the presence of pyrophosphatase, there is sufficient free energy to drive the reaction even at concentrations as low as 1 μM. While we have been unable to search over a broad range of ATP concentrations, we have found that changing the ATP concentration by a factor of 2 does not measurably affect the stoichiometry found.

Fig. 2 shows the results of charging experiments with Tu

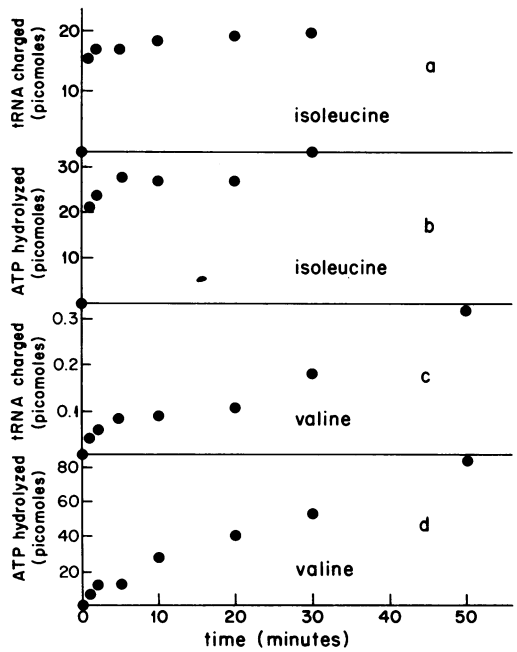

FIG. 2. The time dependence of the amount of tRNA charged and amount of ATP hydrolyzed for charging with isoleucine and valine. (a) tRNA charged, isoleucine; (b) ATP hydrolysis, isoleucine; (c) tRNA charged, valine; (d) ATP hydrolysis, valine. Note the difference in scale for (c) and (d). Conditions were as in Fig. 1, except the appropriate amino acid was at 72 μM, [ATP] = 32 μM, and Tu·GTP was added at a concentration of 2 μM. Aliquot volume was 25 μl, containing about 20 pmol of active $tRNA^{Ile}$.

to prevent the breakdown of aminoacylated tRNA. The experiments were carried out with 2 μM Tu, 32 μM ATP, about 0.8 μM $tRNA^{Ile}$ in unfractionated tRNA, and 72 μM amino acid in all cases. When the amino acid was isoleucine, the charging reaction was very fast, virtually complete within the first minute. The total amount of $Ile\text{-}tRNA^{Ile}$ generated was 19 pmol (Fig. 2a), equal to the total amount of chargeable $tRNA^{Ile}$ in the 25 μl aliquot. The amount of ATP hydrolyzed was simultaneously monitored in the same reaction volume, with results shown in Fig. 2b. Just as in the charging assay, there is a burst of hydrolysis, with no further hydrolysis, within experimental uncertainties, after the first 3 min (30 ATP pmol) was consumed in the charging. The effectiveness of Tu in eliminating the enzymatic deacylation is clear in Fig. 2b, for without Tu there would have been steady ATP hydrolysis, with a net of about 150 pmol consumed in 30 min. When the product is protected with Tu, hydrolysis stops when charging stops, and 1.6 ATP molecules were hydrolyzed by the reaction for each tRNA charged with isoleucine.

The equivalent results with valine substituted for isoleucine are shown in Fig. 2c and d. Charging takes place steadily, and is approximately linear with time, but at a very slow rate, reaching only 1% in 30 min. Phosphate hydrolysis goes on briskly, with no signs of saturating. The stoichiometry of this reaction can be examined by plotting the amount of charging versus the amount of ATP hydrolysis, as in Fig. 3. The time of each sample is indicated next to the experimental point. Within experimental error, the reaction has the same stoichiometry at all times, with 270 mol of ATP hydrolyzed per mol of $Val\text{-}tRNA^{Ile}$ formed. This plot has the advantage of showing the stoichiometry directly and requires no details of kinetics. Errors in the time at which samples are

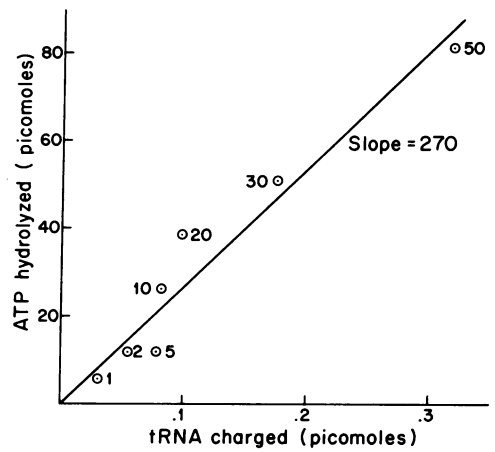

FIG. 3. The relation between tRNA charged and phosphate hydrolyzed for the case of valine. Data are from Fig. 2. The number beside the experimental point shows the time (min) after initiation at which the point was taken.

taken automatically cancel from such a plot because the same aliquot is analyzed for ATP hydrolysis and Val-tRNA^{Ile}.

When the charging has not taken place vigorously (for example, when very low ATP concentrations are used), curves like those of Fig. 2c often fall below linear after long times, indicating that the deacylation reaction [7, 8], while small, is not completely negligible when the forward reaction is too slow. At early times, however, all experiments we have done with the Tu "sink" show a linear relation between ATP consumed and Val-tRNA^{Ile} formed, with the same high stoichiometry of about 250 ATP molecules per Val-tRNA^{Ile} molecule. Doubling the Tu concentration does not affect the result.

Unfractionated tRNA was used in the charging experiments. When charging is performed with isoleucine, only tRNA^{Ile} is charged. There might be more uncertainty whether this was still the case when valine is substituted. To examine this point, charged tRNA described above was fractionated on a reversed-phase chromatography column. Uncharged unfractionated tRNA gives a single very broad peak. When unfractionated tRNA was charged with isoleucine using *E*, three narrow peaks were seen, presumably corresponding to three different kinds of tRNA^{Ile} associated with the different codons for Ile. When valine was substituted for isoleucine, the same peak pattern was seen, with very similar relative areas and peak positions. The small differences between the valine and isoleucine patterns are real and probably due chiefly to the slightly different behavior of Val-tRNA^{Ile} and Ile-tRNA^{Ile} on the column. In general, no major difference in the chromatographic behavior is observed between the acylated and nonacylated tRNA (19), except in certain cases (20). Thus the tRNA that is mischarged (with valine) is by this test the same one that is charged with isoleucine.

DISCUSSION

In the presence of Tu·GTP to prevent the hydrolysis of aminoacylated tRNA, tRNA^{Ile} is charged with isoleucine by the cognate synthetase with the simultaneous hydrolysis of 1.5 ± 0.2 mol of ATP per mol of Ile-tRNA^{Ile} produced. The major source of uncertainty in this number comes from uncertainties in comparative counting efficiencies (because of differences in technique for counting total ATP versus counting released phosphate, or for counting uncharged amino acids and aminoacyl-tRNA). Within the reaction scheme 3 of kinetic proofreading, this number is expected to be greater than or equal to one.

Under the same circumstances, tRNA^{Ile} is charged with valine with the hydrolysis of 270 mol of ATP per mol of Val-tRNA^{Ile} produced. The same fractional uncertainty in the stoichiometry ratio occurs here as for Ile-tRNA^{Ile}. The kinetic proofreading scheme 3 anticipates that a large stoichiometry ratio should occur for incorrect charging. By contrast, charging based on a Michaelis picture (scheme 1, or equivalently 3 without the side-path $C' + c$, or 5 without the side reaction path to E + tRNA^{Ile} + Ile) would not proofread, and would predict a 1:1 stoichiometry for charging with any amino acid.

These stoichiometry ratios imply the following. For every mol of the intermediate E·(Ile-tRNA^{Ile}) formed, 1/1.5 mol of Ile-tRNA^{Ile} will be formed (and correspondingly, 0.5/1.5 mol of Ile + tRNA^{Ile} will be regenerated). But for every mol of the intermediate E·(Val-tRNA^{Ile}) formed, 1/270 mol of Val·tRNA^{Ile} are generated, and 269/270 mol of Val + tRNA^{Ile} are regenerated. Since the reactions to form the intermediates E·(Val·AMP) and E·(Ile·AMP) are essentially irreversible in the presence of pyrophosphatase, the fractional number of errors (mischarging/charging) ≡ f for a reaction with valine and isoleucine simultaneously competing for the enzyme is

$$f = \left[\frac{\text{rate of formation of } E\cdot(\text{Val·AMP})}{\text{rate of formation of } E\cdot(\text{Ile·AMP})}\right]\cdot\left[\frac{1/270}{1/1.5}\right].$$

The first bracket is the effectiveness of selection by the first Michaelis complex, and the irreversible hydrolysis following it. The second bracket is the improvement in overall fidelity due to the proofreading step, and is a factor of about 1/180. (In this *ratio*, uncertainties due to the counting efficiency questions all cancel.)

The effectiveness of the first Michaelis step in discriminating between valine and isoleucine has been studied (21) both by pyrophosphate exchange and by a hydroxamate assay of aminoacyl AMP. In both methods of study the Michaelis constants for these reactions discriminate between valine and isoleucine by a factor of 100, whereas V_{max} for pyrophosphate release is essentially independent of which amino acid is used. Thus, in the presence of equal concentrations of free valine and free isoleucine, the net charging will have an error fraction of

$$f = \left[\frac{1}{100}\right] \times \left[\frac{1}{180}\right] \approx \frac{1}{10{,}000}.$$

The proofreading improvement of the error rate is by about the same factor as the initial discrimination.

Is there a further reduction in the level of errors after the release of charged tRNA by a synthetase molecule? In principle, the enzymatic deacylation reaction allows such a discrimination by the rebinding of charged tRNA^{Ile} to a synthetase molecule, followed by selective release of the mischarged valine (11). On the basis of the *in vivo* turnover rate of tRNA in *E. coli*, known and estimated concentrations of enzyme and Tu in the cell, and extrapolations from data of ref. 11 and our own work, we estimate the probability that a mischarged Val·tRNA^{Ile} released from the enzyme is used in protein synthesis is greater than ½. While the latitude for error in this estimate is considerable, it suggests that error correction after release from the enzyme is relatively unimportant *in vivo*. Nor is it biologically necessary, for the net

accuracy in distinguishing valine from isoleucine in charging is better than one part in 10^4 as a result of kinetic proofreading, and the *in vivo* misincorporation of valine for correct isoleucine in protein synthesis is estimated (1) as 3 in 10^4.

While we have made use of a very simple kinetic scheme for describing the overall chemical reaction, the operation of E is more complicated than the scheme would imply. For example, the release of charged tRNA, often an *in vitro* rate-limiting step, is reported to be facilitated by isoleucine, by isoleucine-AMP, and by $tRNA^{Ile}$ (12, 22). Because of such complications, naive quantitative comparisons of kinetic parameters taken from different experiments must be viewed with caution. Fortunately, the demonstration of proofreading by the stoichiometry measurement does not necessitate the determination of many of the steps. Within the simple kinetic framework, the stoichiometry ratios could be predicted from a complete determination of the Michaelis kinetics of the hydrolysis of Ile-$tRNA^{Ile}$ and Val-$tRNA^{Ile}$ by E. The study at 15° of this reaction by Eldred and Schimmel (11) is not complete enough to do this, but is qualitatively consistent with a large stoichiometry difference between the two cases.

The kinetic scheme **5** can be viewed as a means of proofreading the amino acid by the simple method **3**. The same reaction **5** can also be viewed as a problem in the selection of tRNA, and can also be used to proofread tRNA.

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