# Isoleucyl-tRNA Synthetase from Bakers' Yeast: Variable Discrimination between $tRNA^{Ile}$ and $tRNA^{Val}$ and Different Pathways of Cognate and Noncognate Aminoacylation under Standard Conditions, in the Presence of Pyrophosphatase, Elongation Factor Tu–GTP Complex, and Spermine†

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ABSTRACT: Error rates in discrimination between cognate $tRNA^{Ile}$ and noncognate $tRNA^{Val}$ in the aminoacylation reaction with isoleucine catalyzed by isoleucyl-tRNA synthetase from yeast have been investigated in three sets of experiments under different assay conditions. The overall discrimination factor was first determined by isoleucylation of $tRNA^{Val}$/$tRNA^{Ile}$ mixtures. In the second set of experiments, the number of AMP molecules formed per Ile-tRNA in the cognate and noncognate reactions was measured. The higher AMP formation in the noncognate aminoacylation is assigned to a proofreading reaction step. The calculated proofreading factors and an estimated initial discrimination factor yield overall discriminations that are consistent with those obtained from the first set of experiments. In the third series of studies, the orders of substrate addition and product release of cognate and noncognate isoleucylation reactions were investigated by initial rate kinetic methods. From $k_{cat}$ and $K_m$ values, the overall discrimination factors were calculated and showed again a good coincidence with those observed in the preceding sets of experiments. Besides under standard assay conditions, aminoacylation reactions were studied in the presence of pyrophosphatase or elongation factor Tu–GTP complex, under addition of both these proteins, in presence of these two additional proteins and spermine at high and low magnesium concentrations, and under special conditions that favor misacylations. Furthermore, isoleucylation of $tRNA^{Ile}$ was tested at increased and decreased pH in the standard enzyme assay. Variation of the assay conditions results in changing discrimination factors, which differ by a factor of about 10. Substitution of $tRNA^{Ile}$ by $tRNA^{Val}$ in the isoleucylation reaction causes changes in substrate addition and product release orders and thus of the whole catalytic cycle. For aminoacylation of $tRNA^{Ile}$, four different orders of substrate addition and product release appear: the sequential ordered ter-ter, the rapid equilibrium sequential random ter-ter, the random bi-uni uni-bi ping-pong, and a bi-bi uni-uni ping-pong mechanism with a rapid equilibrium segment. $tRNA^{Val}$ is aminoacylated in rapid equilibrium random ter-ter order, in a bi-bi uni-uni ping-pong mechanism with a rapid equilibrium segment, and in two bi-uni uni-bi ping-pong mechanisms. It is assumed that the different assay conditions can be regarded as a stepwise approximation to physiological conditions and that considerable changes in error rates may be also possible in vivo up to 1 order of magnitude.

One of the major current theories of biological aging is still the error theory, which states that during aging the error frequency of protein biosynthesis may eventually become great enough to impair cell function [e.g., see Bullough (1971), Orgel (1963, 1973), and Hayflick (1975)]. The theory of error catastrophy inspired many investigators to study the substrate specificity of enzymes involved in protein biosynthesis; especially, the ability of aminoacyl-tRNA synthetases to select their cognate substrates was tested extensively [e.g., see Igloi & Cramer (1978), Cramer et al. (1979), and Fersht (1981)]. In considerations of possibly error-induced aging phenomena, also aminoacyl-tRNA synthetases from mutated organisms were included, which showed less specificities for their substrates (Printz & Gross, 1967; Lewis & Holliday, 1970; Orgel, 1973). However, another important point in accuracy studies of protein biosynthesis was somewhat neglected up till now, namely, the variability of error rates occurring in intact wild-type aminoacylation systems under different conditions, which may be similar to variations of the physiological conditions in different stages of the cell cycle.

In the present study, we investigate the variation range of discrimination between $tRNA^{Val}$ and $tRNA^{Ile}$ in the aminoacylation with isoleucine catalyzed by isoleucyl-tRNA synthetase from yeast in three different approaches. In a first simple series of experiments, mixtures of $tRNA^{Val}$ and $tRNA^{Ile}$ are aminoacylated to determine the overall discrimination factor as the quotient of the aminoacylation rates of the two substrates under different conditions. In the second set of studies, the consumption of ATP in the aminoacylation reactions is measured to clear the question whether a hydrolytic correction mechanism exists that diminishes misacylation rates. The aminoacylation reaction is generally written as a two-step mechanism resulting in formation of 1 mol of AMP/mol of aminoacyl-tRNA; consumption of more than one ATP for one

$$E + ATP + aa \rightleftharpoons E\cdot aa\cdot AMP + PP_i$$

$$E\cdot aa\text{-}AMP + tRNA \rightleftharpoons E + aa\text{-}tRNA + AMP$$

aa-tRNA would indicate a proofreading mechanism by which the aminoacyl adenylate formed in the first reaction step or the aminoacyl-tRNA synthesized in the second step is hydrolyzed if a noncognate tRNA is attached to the enzyme. The existence of such a mechanism in aminoacylation of noncognate tRNAs is still a controverse matter (Yamane & Hopfield, 1977; Savageau & Freter, 1979; Fresht, 1979). The "proofreading factors" observed in our experiments and an estimated initial binding factor result in discrimination factors of the same range as obtained in the direct aminoacylation experiments.

In the third part of our studies, the orders of substrate addition and product release are determined for the cognate and the noncognate system by initial rate kinetics, in both cases under the same different conditions as applied before. The experiments run in the noncognate system may be of special interest because a misacylation reaction was not investigated

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before by initial rate kinetics. For two different pathways of the cognate aminoacylation reaction detected by kinetic studies with isoleucyl-tRNA synthetase from yeast (Freist et al., 1981b), different $k_{cat}$ values had been observed, which may implicate also changes in the accuracy achieved by the enzyme. Therefore, attention should also be paid to the possibility of a correlation between accuracy and orders of substrate addition and product release. Furthermore, possible changes in these orders caused by substitution of $tRNA^{Ile}$ by $tRNA^{Val}$ indicate variations of the overall catalytic cycle, which may be important for calculation of discrimination factors from rate constants of single reaction steps in model networks of aminoacylation. From the kinetic constants $k_{cat}$ and $K_m$ obtained in our studies, the discrimination factors for the two substrates $tRNA^{Ile}$ and $tRNA^{Val}$ are also calculated and confirm those obtained from aminoacylations of $tRNA^{Val}/tRNA^{Ile}$ mixtures.

## Experimental Procedures

*Materials.* Isoleucyl-tRNA synthetase (EC 6.1.1.5) was purified from bakers' yeast according to von der Haar (1973); the preparation had a specific activity of 493 units/mg of protein in the aminoacylation reaction. One enzyme unit catalyzed the aminoacylation of 1 nmol of tRNA in 1 min at 37 °C under standard conditions. The preparation did not show any aminoacylation activity with valine and $tRNA^{Val}$. Pure $tRNA^{Ile}_{II}$ and $tRNA^{Val}_{I}$ were isolated from commercially available yeast tRNA (Boehringer Mannheim, FRG) as described by von der Haar & Cramer (1978). The modified tRNAs ($tRNA\text{-}^{Ile}$-C-C-A(3′$NH_2$), $tRNA^{Ile}$-C-C-3′dA, and $tRNA^{Ile}$-C-C-2′dA) were prepared as mentioned in Freist & Cramer (1983). The preparations were analyzed by HPLC[1] with the method described by Bischoff et al. (1983) and found to be free of other tRNA species. Isoleucyl-tRNA synthetase and $tRNA^{Ile}$ from *Escherichia coli* were prepared as outlined by Freist et al. (1982).

Elongation factor Tu was isolated from commercially available *E. coli* MRE 600 cells (E. Merck, Darmstadt, FRG) according to the two-step procedure described by Leberman et al. (1980) with the modifications published by Piechulla & Küntzel (1983). In order to separate the last traces of EF-G from the nearly pure EF-Tu, the active fractions from the previous AcA 44 gel filtration were applied to a Sepharose 4B column (3 × 25 cm) equilibrated with 60% ammonium sulfate in 50 mM Tris-HCl buffer, pH 7.5, containing 10 μM GDP, 10 mM $MgSO_4$, 20 μM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol, and 5% v/v glycerol. The column was eluted with a linear gradient of 50–25% ammonium sulfate according to the reversible salting-out procedure described in von der Haar (1979). EF-Tu is eluted with 32% ammonium sulfate. The active fractions were combined, and an equal volume of saturated ammonium sulfate solution was added. The precipitate was collected by centrifugation and dissolved in the buffer mixture mentioned above without addition of ammonium sulfate. For storage at −20 °C, an equal volume of glycerol was added. The specific activity of the pure EF-Tu was 28 400 units/mg. There was no loss of activity during storage at −20 °C during more than 3 years.

Inorganic pyrophosphatase (EC 3.6.1.1) with a specific activity of 635 units/mg of protein was obtained from Sigma Chemical Co. (St. Louis, MO). $^{14}C$-Labeled isoleucine with a specific activity of 342 mCi/mmol was purchased from the Radiochemical Centre (Amersham, Great Britain). To check this [$^{14}C$]isoleucine for traces of valine, which could disturb misacylation experiments, it was added in the normal concentration into an aminoacylation mixture containing $tRNA^{Val}$ and valyl-tRNA synthetase (EC 6.1.1.9). No incorporation of the $^{14}C$ label into $tRNA^{Val}$ could be detected. Pyruvate kinase from rabbit muscle, phosphoenolpyruvate, guanosine 5′-triphosphate (GTP), and 5′-guanylyl imidodiphosphate (GMP-PNP) were products of Boehringer Mannheim (Mannheim, FRG). Spermine was obtained from Serva Feinbiochemica (Heidelberg, FRG). Isoleucinol (DL-2-amino-3-methylpentanol) was a product of EGA Chemie (Steinheim, FRG). [8-$^{14}C$]Adenosine 5′-triphosphate with a specific activity of 546 mCi/mmol was purchased from Amersham Buchler GmbH (Braunschweig, FRG) and was diluted with a 10-fold amount of unlabelled material; for thin-layer chromatography, PEI–cellulose F sheets from Merck AG (Darmstadt, FRG) were used.

*Aminoacylation Assay.* The velocity of the esterification reaction was measured by the amount of [$^{14}C$]isoleucine incorporated into tRNA as described by Faulhammer & Cramer (1979). The standard reaction mixture (0.1 mL) contained 0.15 M Tris-HCl buffer, pH 7.65, 0.1 M KCl, and 0.015 M $MgSO_4$; concentrations of substrates were 0.5 mM ATP, 0.023 mM [$^{14}C$]isoleucine, 0.002 mM $tRNA^{Ile}$, and 0.0014 mM $tRNA^{Val}$ when added in fixed concentrations (for kinetics, see below). In experiments with inorganic pyrophosphatase, 0.25 unit of this enzyme was added to 0.1 mL of the reaction mixture. For kinetics in the presence of EF-Tu–GTP, this complex was regenerated from EF-Tu–GDP in a mixture consisting of 0.1 M Tris-HCl buffer, pH 7.65, 0.1 M KCl, 0.02 M $MgSO_4$, 0.8 mM phosphoenolpyruvate, 800 μg/mL pyruvate kinase, 0.08 mM GTP and also 0.035 mM GMP-PNP, and 0.015 mM EF-Tu–GTP by incubation at 37 °C for 10 min. A 0.1-mL sample of the aminoacylation mixture contained 25 μL of this EF-Tu–GTP regeneration solution. If spermine was present in the aminoacylation mixture, its concentration was 1 mM. For aminoacylation at pH 9, the mixture consisted of 0.01 M Tris-HCl buffer, pH 9, 0.008 M $MgSO_4$, and again the appropriate concentrations of ATP, [$^{14}C$]isoleucine, and tRNA. Experiments at pH 6.5 were carried out in 0.15 M PIPES buffer (Merck AG, Darmstadt, FRG).

*Inorganic Pyrophosphatase Assay.* Inorganic pyrophosphatase was assayed in 0.4 M Tris-HCl (pH 7.65), containing 20 mM $MgSO_4$ and 3 mM pyrophosphatase. Spots were analyzed by Hanes' reagent (Hanes & Isherwood, 1949).

*Aminoacylation of $tRNA^{Ile}/tRNA^{Val}$ Mixtures.* $tRNA^{Ile}/tRNA^{Val}$ mixtures were aminoacylated in the aminoacylation assays and in the presence of the additives given above. The total amount of tRNA was kept constant at 0.8 μM, while aminoacylation rates were measured with pure $tRNA^{Ile}$ and $tRNA^{Val}$ and seven different mixtures in which the quotients $[tRNA^{Ile}]/[tRNA^{Val}]$ were 7.0, 3.0, 1.7, 1.0, 0.6, 0.3, and 0.14. Aminoacylation rates were linearly decreasing with decreasing $tRNA^{Ile}$ concentration, and reaction rates for acylation of pure $tRNA^{Ile}$ and $tRNA^{Val}$ at 0.8 μM concentration were extrapolated and used for calculation of discrimination factors according to the equation $V_{tRNA^{Ile}}/v_{tRNA^{Val}} = ([tRNA^{Ile}]/[tRNA^{Val}])D$.

*AMP Formation under Aminoacylation Conditions.* The assay mixture contained the same constituents as in the aminoacylation assay; the concentration of [$^{14}C$]ATP was 0.2 mM, of [$^{14}C$]isoleucine was 0.02 mM, and of $tRNA^{Ile}$ or $tRNA^{Val}$ was 0.007 mM. For measurement of the aminoacylation rates, samples of 0.05 mL were taken in intervals

[1] Abbreviations: HPLC, high-pressure liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PIPES, 1,4-piperazinediethanesulfonic acid.

of 0.5 min and treated as in Faulhammer & Cramer (1979). For determination of AMP formation rate, samples of 0.05 mL were spotted onto PEI–cellulose F sheets in intervals of 0.5 min. Unlabeled ATP, ADP, and AMP were applied to the plates at the origin prior to use. The nucleotides were separated by ascending chromatography for 90 min with a mixture of 1 M LiCl in 1 M acetic acid and 2-propanol (2:1 v/v). After being dried, the spots were marked in UV light and cut out, and the radioactivity was counted in a liquid scintillation counter. Rates of AMP formation were calculated by computerized linear regression with an experimental error of about 10%. The stoichiometry of AMP formation is given as the quotient of rate of AMP formation and aminoacyl-tRNA formation.

*Kinetics.* The nomenclature of kinetics is that of Cleland (1963a–c, 1970) and Fromm (1975). All data were analyzed in the form of primary plots, which were obtained by plotting the reciprocal of the initial velocity against the reciprocal of the variable substrate concentration in the presence of changing fixed levels of a second substrate or inhibitor. From these plots, kinetic patterns were determined (competitive, noncompetitive, uncompetitive). For control, data were also plotted in Woolf–Augustinssons–Hofstee plots (initial velocity against the quotient obtained from initial velocity and substrate concentration) (Segel, 1975a). In every case, the same types of kinetic pattern were observed. In all initial velocity studies, conditions were selected to ensure linear reaction rates.

When ATP was used as variable substrate, its concentration was varied in the range of 10–50 $\mu$M, the isoleucine concentration was 1–6 $\mu$M, the $tRNA^{Ile}$ concentration was 0.1–1.0 $\mu$M, and the concentration of $tRNA^{Val}$ was varied in the range of 0.3–0.8 $\mu$M. Inhibition types were determined from intersection points of two or three Lineweaver–Burk plots (see paragraph at end of paper regarding supplementary material), one representing kinetics in the absence and one or two in the presence of an inhibitor. Concentrations or inhibitors were in the range of their $K_i$ values (compare Tables III and IV). In bisubstrate kinetics, patterns were again determined from two or three Lineweaver–Burk plots; the concentrations of the changing fixed substrates were in the range of their $K_m$ values (Table II). Each plot was obtained from six test data for which the experimental error was estimated to be in the range of 10%. The reaction mixture was preincubated at 37 °C; then the enzyme was added. Samples of 0.02 mL were taken in intervals of 0.25, 0.5, or 1 min. The concentration of active enzyme was for aminoacylation of $tRNA^{Ile}$ 7–10 pM; for aminoacylation of $tRNA^{Val}$, it was 10–70 pM.

## Results

*Discrimination in Aminoacylation of $tRNA^{Ile}/tRNA^{Val}$ Mixtures.* $tRNA^{Val}$ belongs to a family of tRNAs specific for hydrophobic amino acids that are separated from $tRNA^{Ile}$ by the lowest intercognate barriers (Yarus & Mertes, 1973). The nucleotide sequences of $tRNA^{Ile}$ and three $tRNA^{Val}$ isoacceptors from yeast exhibit differences in all stem regions and loops of the cloverleaf structure [see Gauss & Sprinzl (1983)]; however, in some parts of the tRNA molecule that have been proposed as recognition sites, $tRNA^{Ile}$ and $tRNA^{Val}$ show similarities. These are the fourth nucleotide of the C-C-A end ("discriminator base"), the first three base pairs of the C-C-A stem, and the relatively big-sized dihydrouridine loop. In a recognition system based on a binary code, $tRNA^{Val}$ and $tRNA^{Ile}$ also show the lowest difference (Freist & Cramer, 1976). In misacylation experiments, the $tRNA^{Val}$ species must be completely free of $tRNA^{Ile}$. Our preparations, which turned out to be free of $tRNA^{Ile}$ by HPLC analysis, were chargeable with isoleucine by isoleucyl-tRNA synthetase with plateau values up to 12% of the whole tRNA, whereas in aminoacylation with valine by valyl-tRNA synthetase the plateau of the reaction was reached when 90% of the tRNA was converted to the aminoacyl ester. The observed extent of misacylation is in a range as often described for heterologous systems (Giegé et al., 1971, 1972, 1974; Kern et al., 1972; Ebel et al., 1973; Yarus & Mertes, 1973; Ritter et al., 1970; Taglang et al., 1970). To exclude the possibility that misacylation of the $tRNA^{Val}$ preparation was caused by a $tRNA^{Ile}$ isoacceptor, which could not be separated during the preparation procedure and even not in HPLC analysis, the $tRNA_I^{Val}$ preparation was additionally incubated with a mixture of [$^3$H]valine and [$^{14}$C]isoleucine and valyl- and isoleucyl-tRNA synthetase. In this control experiment, only [$^3$H]valine was incorporated into the aminoacylation product, proving definitively the absence of $tRNA^{Ile}$.

In our in vitro experiments naturally one cannot obtain the discrimination factors that appear under physiological conditions. As pointed out by Ebel et al. (1973) the level of error should be already diminished when all tRNA species are present. If also the whole set of aminoacyl-tRNA synthetases is present, the error level is further minimized as described for a network of two tRNA species and two aminoacyl-tRNA synthetases (Yarus, 1972). In fact, misacylation appears to be completely suppressed for our detection methods if the $tRNA^{Val}$ preparation was incubated with unlabeled valine, $^{14}$C-labeled isoleucine, and valyl-tRNA and isoleucyl-tRNA synthetases.

For discrimination between the cognate $tRNA^{Ile}$ and the noncognate $tRNA^{Val}$, the quotient obtained from the aminoacylation rates of the two tRNAs is determinant for error rates and is given by the equation (Fersht, 1977)

$$v_{tRNA^{Ile}}/v_{tRNA^{Val}} = ([tRNA^{Ile}]/[tRNA^{Val}])e^{-\Delta\Delta G_b^{\ddagger}/(RT)}$$

The "discrimintion factor" $D = e^{-\Delta\Delta G_b^{\ddagger}/(RT)}$, in which $\Delta\Delta G_b^{\ddagger}$ is the difference between the free binding enthalpies of the two substrates in the transition state, can be easily calculated from the aminoacylation rates of the two tRNAs. In eight experiments, aminoacylation rates of different $tRNA^{Ile}/tRNA^{Val}$ mixtures were measured under different conditions, $v_{tRNA^{Ile}}$ and $v_{tRNA^{Val}}$ were determined by extrapolation of the linearly decreasing rates to zero concentration of the other tRNA, and discrimination factors were calculated as the quotient of the reaction rates (Table I).

First, the experiment was run under assay conditions that are normally used for aminoacyl-tRNA synthetases and are optimized for highest enzyme activity. For tRNA concentrations lower than 1 $\mu$M, a discrimination factor of 75 was found (Table I). At higher tRNA concentrations, discrimination slows down to the very low value of 2 that is due to an inhibition by $tRNA^{Val}$, which in this case is present in a concentration up to 8-fold as high as its $K_m$ value (Table II). In the next experiment, inorganic pyrophosphatase was added, which eliminates the reaction product $PP_i$ from the reaction mixture. This enzyme is one of the most common enzymes in *E. coli* (Josse & Wong, 1971) or yeast (Butler, 1971); however, in spite of high concentrations of this enzyme, also high levels of pyrophosphate have been reported in *E. coli* (Kukko & Heinonen, 1982) and yeast (Ermakova et al., 1981), and its mode of escape from pyrophosphatase action has not been clarified (Josse & Wong, 1971). The discrimination factor observed in presence of pyrophosphatase was 119 (Tables I and II).

The following accuracy tests were carried out in the presence of elongation factor Tu–GTP complex from *E. coli*, which has

Table I: Aminoacylation Rates at [tRNA] = 0.8 μM Determined from Mixing Experiments and Resulting Discrimination Factor $D$, AMP Formation in Cognate and Noncognate Aminoacylation, Proofreading Factor $P$, and Calculated Initial Discrimination Factor $I$

| conditions | mixing experiments | | | AMP formation | | | |
|---|---|---|---|---|---|---|---|
| | $v_{tRNA^{Ile}}$ (pmol min$^{-1}$) | $v_{tRNA^{Val}}$ (pmol min$^{-1}$) | $D = v_{tRNA^{Ile}}/v_{tRNA^{Val}}$ | molecules of AMP formed/ molecule of Ile-tRNA$^{Ile}$ | molecules of AMP formed/ molecule of Val-tRNA$^{Ile}$ | $P$ | $I$ |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$ | 3.0 | 0.04 | 75 | 5.5 | 154 | 34 | 2.6 |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$, PPase | 2.38 | 0.02 | 119 | 6.3 | 177 | 33 | 4.2 |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$, EF-Tu-GTP | 2.40 | 0.04 | 60 | 3.0 | 165 | 83 | 1.1 |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$, EF-Tu-GTP, PPase | 1.90 | 0.11 | 17 | 5.1 | 73 | 18 | 1.1 |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$, EF-Tu-GTP, PPase, spermine | 2.90 | 0.15 | 19 | 6.1 | 81 | 16 | 1.1 |
| pH 7.65, 0.1 M KCl, 1 mM $Mg^{2+}$, EF-Tu-GTP, PPase, spermine | 1.84 | 0.06 | 31 | 3.4 | 86 | 36 | 1.2 |
| pH 8.6, 0.1 M KCl, 15 mM $Mg^{2+}$ | | | | 6.9 | 823 | 139 | |
| pH 6.5, 0.1 M KCl, 15 mM $Mg^{2+}$ | | | | 3.5 | 282 | 113 | |
| pH 9.0, 8 mM $Mg^{2+}$ | 0.80 | 0.60 | 1.3 | ~10 | ~10 | 1.3 | 1.3 |

been found to be present in sufficient amounts to bind all aminoacylated tRNA in this organism. Although isolated from another source, this elongation factor Tu complexes also aminoacyl-tRNAs from yeast (Furano, 1975; Pingoud et al., 1977; Pingoud & Urbanke, 1980; Tanada et al., 1981, 1982). The discrimination factor found in the presence of EF-Tu–GTP complex in the normal aminoacylation assay was 60 (Tables I and II). When pyrophosphatase was also added to the reaction mixture, the factor slowed further down to 17 (Tables I and II). As a third additional compound, spermine was added to the aminoacylation assay. This substance is one of the most common polyamines occurring in all plant and animal cells (Sakai & Cohen, 1976). The influence of spermine cations on the tRNA structure and on the action of aminoacyl-tRNA synthetases was studied extensively (Sakai & Cohen, 1976; Loftfield et al., 1981), and changes of order of substrate addition and product release were reported (Freist et al., 1982; Takeda & Matsuzaki, 1974; Igarashi et al., 1978; Natarajan & Gopinathan, 1981). The discrimination factor observed in presence of spermine was 19 and practically not higher than that in the presence of EF-Tu–GTP and pyrophosphatase (Tables I and II).

In the standard enzyme assay for aminoacyl-tRNA synthetases, $Mg^{2+}$ concentrations higher than the physiological one of about 1 mM (Loftfield et al., 1981; Veleso et al., 1973) are applied to get optimal reaction rates. To test a possible influence of lowering the $Mg^{2+}$ concentration, the discrimination factor was also determined in the presence of EF-Tu–GTP, pyrophosphatase, spermine, and 1 mM $Mg^{2+}$. The factor was found to be somewhat higher with a value of 31 (Tables I and II).

As a last variation of the enzyme assay in these experiments, "misacylation conditions" were used that had been applied to achieve acylations of noncognate tRNAs (Giegé et al., 1972, 1974; Kern et al., 1972; Ebel et al., 1973). In this reaction mixture (pH 9, 8 mM $MgSO_4$), nearly no discrimination between the two tRNAs could be observed; the discriminatioin factor was determined as 1.3 (Tables I and II).

*Calculation of Discrimination Factors from AMP Formation.* According to Yamane & Hopfield (1977) in misacylation of tRNA$^{fMet}$ and tRNA$^{Phe}$ with isoleucyl-tRNA synthetase from *E. coli*, 25 and 40 molecules of ATP are hydrolyzed for each Ile-tRNA$^{fMet}$ and Ile-tRNA$^{Phe}$. Also, Savageau & Freter (1979) calculated proofreading factors for misacylation reactions from literature data, whereas Fersht (1979) regarded the observed AMP formation as an artifact generated by slow decomposition of the enzyme–aminoacyl adenylate complex. To check the misacylation of tRNA$^{Val}$ by isoleucyl-tRNA synthetase from yeast, AMP formation by the cognate and noncognate aminoacylation reactions was studied. Hopfield et al. (1976) found that 1.5 molecules of ATP were consumed for each Ile-tRNA$^{Ile}$ in the aminoacylation of this tRNA catalyzed by isoleucyl-tRNA synthetase from *E. coli*, whereas Fersht & Kaethner (1976) found an even lower value of 1.3 mol of ATP hydrolyzed/mol of Ile-tRNA$^{Ile}$. When we repeated these measurements of ATP consumption with isoleucyl-tRNA synthetase from *E. coli* in the presence of elongation factor EF-Tu–GTP, we observed also an ATP consumption of 1.5 mol for 1 mol of Ile-tRNA$^{Ile}$. However, when the experiment was run with tRNA$^{Ile}$ and isoleucyl-tRNA synthetase from yeast (but without addition of EF-Tu–GTP complex), ATP consumption was considerably higher with 5.5 mol of ATP for 1 mol of Ile-tRNA$^{Ile}$ (Table I). Lower values (1.75, 1.4, or 1.25 mol) similar as with the enzyme from *E. coli* were obtained when modified tRNAs such as tRNA$^{Ile}$-C-C-3′dA and tRNA$^{Ile}$-C-C-A(3′$NH_2$) were isoleucylated, an observation that excludes the possibility that the high amounts of AMP are generated by other enzymes with ATPase activity present as impurities in the isoleucyl-tRNA synthetase preparation. The modified tRNAs differ from the natural tRNA$^{Ile}$-C-C-A in their ability to be mischarged with valine because, by lack of the 3′-OH group or substitution of this group by an amino group, a proofreading function of the enzyme is lost that normally prevents acylation with valine (von der Haar & Cramer, 1978). From our experiments on ATP consumption of isoleucyl-tRNA synthetase from yeast, the assumption can be made that in aminoacylation of tRNA$^{Ile}$-C-C-A the hydrolytic proofreading activity is partly directed against the cognate substrate isoleucine, which causes the surprisingly high consumption of ATP in the normal isoleucylation of tRNA$^{Ile}$-C-C-A. When tRNA$^{Ile}$-C-C-2′dA is added to the reaction mixture instead of tRNA$^{Ile}$-C-C-A, AMP formation occurs at the same rate as that with tRNA$^{Ile}$-C-C-3′dA although tRNA$^{Ile}$-C-C-2′dA is not aminoacylated because the accepting 2′-hydroxy group is lacking (Sprinzl & Cramer, 1975; Chinault et al., 1977). This observation is consistent with an assumption made already earlier that a proofreading step that hydrolyzes misactivated valine occurs in the enzyme–adenylate complex before the transfer step of the aminoacylation reaction occurs (Fersht, 1977; Freist & Cramer, 1983).

The overall discrimination between tRNA$^{Val}$ and tRNA$^{Ile}$ is determined by an "initial discrimination factor $I$" and the "proofreading discrimination $P$" (Savageau & Freter, 1979; Yamane & Hopfield, 1977). The initial discrimination is caused by the different affinities of the two tRNA species to the enzyme. Association constants of tRNA$^{Ile}$ and tRNA$^{Val}$ for enzyme–tRNA complexes measured by fluorescence quenching methods with isoleucyl-tRNA synthetase from *E. coli* showed only a small difference in binding of the two

Table II: Conditions, in Aminoacylation Assays, $K_m$ Values of Substrates, $k_{cat}$ Values, Order of Substrate Addition and Product Release, and Discrimination Factors in Aminoacylation of $tRNA^{Ile}$ and $tRNA^{Val}$ with Isoleucine and Isoleucyl-tRNA Synthetase[a]

| | cognate aminoacylation | | | | noncognate aminoacylation | | | | discrimination factor $D^d$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| conditions | substrates | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | order of substrate addition and product release | substrates | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | order of substrate addition and product release | calcd | found |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$ | ATP<br>Ile<br>$tRNA^{Ile}$ | 0.3<br>0.02<br>0.002, 0.0003[b] | 0.83<br>0.64[b] | A B C P Q R | ATP<br>Ile<br>$tRNA^{Val}$ | 0.2<br>0.005<br>0.001 | 0.030 | A, B, C → E → P, Q, R | 14<br>71[b] | 2<br>75[b] |
| pH 7.65, 0.1 M KCl, 50 mM $Mg^{2+}$, PPase | ATP<br>Ile<br>$tRNA^{Ile}$ | 0.5<br>0.04<br>0.002, 0.0003[b] | 1.40<br>0.83[b] | Q + A B P C R; B A | ATP<br>Ile<br>$tRNA^{Val}$ | 0.02<br>0.0014<br>0.002 | 0.054 | P + B A Q C R | 26<br>102[b] | 119[b] |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$, EF-Tu–GTP | ATP<br>Ile<br>$tRNA^{Ile}$ | 0.2<br>0.02<br>0.0005 | 0.28 | A B C P Q R | ATP<br>Ile<br>$tRNA^{Val}$ | 0.04<br>0.003<br>0.003 | 0.027 | A, B, C → E → P, Q, R | 62 | 60 |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$, EF-Tu–GTP, PPase | ATP<br>Ile<br>$tRNA^{Ile}$ | 0.6<br>0.05<br>0.0004 | 0.23 | A B C P Q R | ATP<br>Ile<br>$tRNA^{Val}$ | 0.07<br>0.002<br>0.001 | 0.023 | A, B, C → E → P, Q, R | 25 | 17 |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$, EF-Tu–GTP, PPase, spermine | ATP<br>Ile<br>$tRNA^{Ile}$ | 0.1<br>0.02<br>0.0002 | 0.098 | P + B A Q C R | ATP<br>Ile<br>$tRNA^{Val}$ | 0.02<br>0.002<br>0.001 | 0.023 | A B P? C R Q? | 21 | 19 |
| pH 7.65, 0.1 M KCl, 1 mM $Mg^{2+}$, EF-Tu–GTP, PPase, spermine | ATP<br>Ile<br>$tRNA^{Ile}$ | 0.3<br>0.008<br>0.0006 | 0.135 | P + B A Q C R | ATP<br>Ile<br>$tRNA^{Val}$ | 0.05<br>0.002<br>0.003 | 0.020 | A B P? C R Q? | 34 | 31 |
| pH 9.0, 8 mM $Mg^{2+}$ | ATP<br>Ile<br>$tRNA^{Ile}$ | 0.5<br>0.02<br>0.002 | 0.01 | A B C P Q R | ATP<br>Ile<br>$tRNA^{Val}$ | 0.09<br>0.003<br>0.001 | 0.005 | A + B P C R Q | 1 | 1.3 |
| pH 8.60, 0.1 M KCl, 15 mM $Mg^{2+}$ | ATP<br>Ile<br>$tRNA^{Ile}$ | 0.2<br>0.02<br>0.0003 | 0.52 | A B C P Q R | | | | | 104[c] | |
| pH 6.5, 0.1 M KCl, 15 mM $Mg^{2+}$ | ATP<br>Ile<br>$tRNA^{Ile}$ | 0.2<br>0.03<br>0.0003 | 0.47 | A, B, C → E → P, Q, R | | | | | 94[c] | |

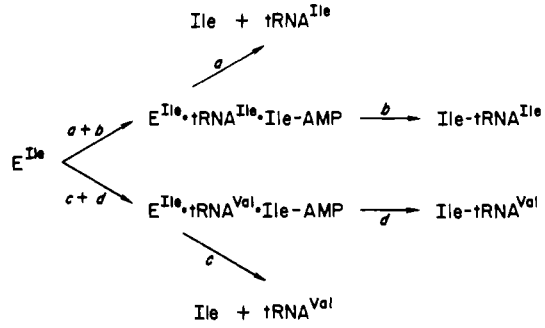
[a] $K_m$ and $k_{cat}$ values are averages from all the experiments. In the illustration of substrate addition and product release, A = ATP, B = isoleucine, C = $tRNA^{Ile}$, P = pyrophosphate, Q = Ile-tRNA, and R = AMP. [b] [tRNA] < 1 μM; this is about one-tenth of usual tRNA. [c] Calculated with averages of *Km* values for $tRNA^{Val}$ and of $k_{cat}$ values for isoleucylation of $tRNA^{Val}$. [d] $D = k_{cat\,tRNA^{Ile}} K_{m\,tRNA^{Val}} / (K_{m\,tRNA^{Ile}} k_{cat\,tRNA^{Val}})$.

tRNAs; the association constant of the cognate $tRNA^{Ile}$ ($10^8$ $M^{-1}$) was only about 5 times higher than that observed for $tRNA^{Val}$ ($2 \times 10^7$ $M^{-1}$) (Lam & Schimmel, 1975). From these data, a low initial discrimination on the order of magnitude as the quotient 5 obtained from the two constants can be expected when calculated from the overall and the proofreading discriminations.

According to Savageau & Freter (1979), an overall discrimination *D* (reciprocal of netto error) is defined as the sum of cognate and noncognate tRNA fluxes divided by the flux of noncognate tRNA, in our case always calculated for the same concentrations of the competing substrates (Scheme I). The initial discrimination factor *I* is represented by the ratio of input flux of $tRNA^{Ile}$ to that of $tRNA^{Val}$ into the reaction network (Scheme I), in our work again for the same concentrations of the two tRNAs. The proofreading factor *P* is defined as the ratio of recycled incorrect flux to that continuing on divided by the ratio of correct flux recycled to that continuing on, measured again for the same tRNA concentrations (Scheme I).

For calculation of the proofreading factor *P*, first the amount of AMP generated in cognate aminoacylation under the different conditions had to be determined. The observed values were in the range from 3 up to 10 molecules of ATP hydrolyzed for one Ile-$tRNA^{Ile}$ formed, under most conditions about six (Table I). In the misacylation reaction the ratio of AMP

Scheme I: Network of Misacylation according to Savageau & Freter (1979)[a]




[a] $D = (b + d)/d$. $I = (a + b)/(c + d)$. $P = (c/d)(b/a)$.

to Ile-tRNA$^{Val}$ differed from 10 up to more than 800 (Table I). The resulting proofreading factors differ from 1 to 140. The factor $P = 1$ occurs under conditions that were especially used to achieve misacylation (Giegé et al., 1972, 1974; Kern et al., 1972; Ebel et al., 1973) and shows that proofreading does not occur under these conditions, which gives also an explanation for the suitability of these assay conditions for noncognate aminoacylation. The discrimination between tRNA$^{Val}$ and tRNA$^{Ile}$ is in this case only determined by the initial discrimintion. The lowest values of proofreading ($P$ = 16) are observed in those cases in which the overall discrimination $D$ was also found to be very low in the experiments with mixtures of tRNA$^{Val}$ and tRNA$^{Ile}$ described above (Table I). The initial discrimination $I$ calculated from the $D$ values obtained in the mixing experiments and the proofreading factor $P$ according to the network conditions of Scheme I differ from 1.1 up to 4.2 and thus are in the order of magnitude as expected from the values of association constants (Table I).

*Initial Rate Kinetics. (1) Aminoacylation of tRNA$^{Ile}$.* In our previous work on isoleucyl-tRNA synthetase from bakers' yeast, two pathways of aminoacylation were detected depending on the presence of pyrophosphatase in the aminoacylation assay (Freist et al., 1981b); in the first case the order of substrate addition and product release is sequential ordered ter-ter, and in the second case a bi-uni uni-bi ping-pong mechanism is observed (Table III). These experiments were only carried out under the normally used assay conditions, which are optimized to obtain greatest enzyme activities. Different $k_{cat}$ values of the two pathways suggested already different accuracies in misacylation reactions. To calculate the overall discrimination from $k_{cat}$ and $K_m$ values and to detect differences in the catalytic cycle, the pathways of cognate and noncognate aminoacylation were further investigated under the conditions applied in the preceding sections.

To study a possible influence of elongation factor Tu–GTP complex on the aminoacylation mechanism, we carried out inhibition experiments with 3′-deoxyadenosine 5′-triphosphate as an "indicator inhibitor" with respect to the three substrates. The inhibition patterns caused by 3′dATP were typical for the sequential ordered ter-ter mechanism (Table III) as observed in previous studies (Freist et al., 1981b); however, the $K_m$ of tRNA$^{Ile}$ is lowered to 0.0005 mM, a value that is very similar to that one observed in experiments with modified tRNA$^{Ile}$s (Freist & Cramer, 1983). The $k_{cat}$ value is also considerably lowered to 0.28 $s^{-1}$. Addition of inorganic pyrophosphatase to the Tu–GTP-containing assay did not change this observation (Table III).

To elucidate the reaction mechanism of isoleucyl-tRNA synthetase in the presence of EF-Tu–GTP complex, pyrophosphatase, and spermine, which are all present under physiological conditions, more detailed inhibition studies, bisubstrate kinetics, and product inhibition had to be carried out. When 3′dATP and isoleucinol were used as inhibitors, the same inhibition patterns were observed. Both were competitive inhibitors when ATP or isoleucine was the variable substrate (Table III) and uncompetitive when the tRNA concentration was varied (Table III).

Table III: Patterns of Bisubstrate Kinetics and Inhibition by Products and Inhibitors in the Aminoacylation of tRNA$^{Ile}$ with Isoleucine and Isoleucyl-tRNA Synthetase under Different Conditions and $K_i$ Values of Inhibitors

| conditions | changing fixed substrate (a), product (b), or inhibitor (c) | variable substrate[a] ATP | Ile | tRNA$^{Ile}$ | $K_i$ (mM) |
|---|---|---|---|---|---|
| pH 7.65, 0.1 M KCl, 50 mM $Mg^{2+}$ | (c) 3′dATP | uc | c | nc | 0.2 |
| pH 7.65, 0.1 M KCl, 50 mM $Mg^{2+}$, PPase | (c) 3′dATP | c | nc | uc | 0.5 |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$, EF-Tu–GTP | (c) 3′dATP | uc | c | nc | 0.1 |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$, EF-Tu–GTP, PPase | (c) 3′dATP | uc | c | nc | 0.05 |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$, EF-Tu–GTP, PPase, spermine | (a) Ile | r | | | |
| | (a) tRNA$^{Ile}$ | | i | | |
| | (b) AMP | c | c | nc | 16.0 |
| | (c) 3′dATP | c | c | uc | 1.6 |
| | (c) isoleucinol | c | c | uc | 80.0 |
| pH 7.65, 0.1 M KCl, 1 mM $Mg^{2+}$, EF-Tu–GTP, PPase, spermine | (c) 3′dATP | c | c | uc | 1.3 |
| pH 9.0, 8 mM $Mg^{2+}$ | (c) 3′dATP | uc | c | nc | 0.1,[b] 0.2[c] |
| pH 6.50, 0.1 M KCl, 15 mM $Mg^{2+}$ | (c) 3′dATP | nc | c | nc | 0.3 |
| pH 8.60, 0.1 M KCl, 15 mM $Mg^{2+}$ | (c) 3′dATP | uc | c | nc | 0.4 |

[a] c, competitive; nc, noncompetitive; uc, uncompetitive; r, reversibly connected; i, irreversibly connected. [b] Slope. [c] Intercept.

At first glance it seems to be unusual that a structural analogue of ATP and an analogue of isoleucine cause the same effects. However, it has already been noticed that some ATP analogues can compete with the amino acid and can cause the same inhibition patterns as analogues of this substrate under the standard conditions (Freist et al., 1981a,b, 1982). This observation may become easily plausible regarding data obtained with crystalline tyrosyl-tRNA synthetase from *Bacillus stearothermophilus*, which binds ATP or AMP also in the tyrosyl binding pocket (Monteilhet & Blow, 1978). Therefore, the inhibition patterns caused by 3′dATP and isoleucinol may together indicate a mechanism with a rapid equilibrium segment in which isoleucine and ATP are added to the enzyme in sequential order with isoleucine first (Table II). In this case, the two inhibitors show competitive inhibition with respect to the two small substrates (Segel, 1975b). The uncompetitive inhibition type with respect to tRNA$^{Ile}$ indicates a product release step between addition of the two small substrates and the tRNA or addition of tRNA prior to ATP and isoleucine. The first possibility is confirmed by bisubstrate kinetics. When isoleucine is the changing fixed substrate and ATP is the

variable one, the reciprocal plots intersect at a point to the left of the vertical axis (Table III) and indicate that ATP and isoleucine bind to enzyme forms, which are reversibly connected. When $tRNA^{Ile}$ is the changing fixed substrate and isoleucine is the variable substrate, parallel patterns are observed that show that between addition of the small substrate and addition of the tRNA a product must be released from the enzyme (Table III). The product AMP causes competitive inhibition when ATP or isoleucine is the variable substrate and noncompetitive patterns when the tRNA concentration is varied (Table III). These observations show that AMP is released before addition of isoleucine and ATP and after addition of the tRNA. The results of these initial rate kinetic studies are consistent with a bi-bi uni-uni ping-pong mechanism. In a rapid equilibrium segment, isoleucine is added first followed by ATP, then the products pyrophosphate and isoleucyl-tRNA are released in unknown order, thereafter, the uncharged tRNA is added, and then AMP dissociates from the enzyme (Table II). The release of an aminoacylated tRNA before addition of the uncharged tRNA can only be understood by regarding the whole catalytic cycle as pointed out extensively in our previous work (Freist & Cramer, 1983). In this mechanism, which was not described before for isoleucyl-tRNA synthetase from yeast, the $K_m$ value of $tRNA^{Ile}$ is 0.0002 mM; the $k_{cat}$ is lowered to 0.098 $s^{-1}$.

To test a possible influence of lowering the $Mg^{2+}$ concentration, the aminoacylation of $tRNA^{Ile}$ in presence of EF-Tu–GTP, pyrophosphatase, and spermine was also conducted at a $Mg^{2+}$ concentration of 1 mM. Inhibition studies with 3′dATP as inhibitor resulted in the same patterns as observed before (Table III) when the experiments were run at 15 mM $Mg^{2+}$. Therefore, it may be concluded that the bi-bi uni-uni ping-pong mechanism was not changed by lowering the $Mg^{2+}$ concentration. The $K_m$ of $tRNA^{Ile}$ was 0.0006 mM; $k_{cat}$ was 0.135 $s^{-1}$.

For a kinetic analysis under "misacylation conditions" (pH 9, 8 mM $MgSO_4$), again inhibition studies with 3′dATP were carried out. The inhibition types showed that the sequential ordered ter-ter mechanism that was observed under standard conditions was not changed under these special conditions (Table III). The $K_m$ value of the tRNA remained also unchanged at 0.002 mM; the $k_{cat}$ value, however, was remarkably lower at 0.01 $s^{-1}$.

To test the influence of changes in pH values under standard conditions, the pH of the Tris buffer was increased to 8.6. Inhibition experiments with 3′dATP showed the same patterns as observed in the sequential ordered ter-ter mechanism (Table III). The $k_{cat}$ value was lowered to 0.52 $s^{-1}$; the apparent $K_m$ of $tRNA^{Ile}$ is 0.0003 mM.

When the pH of the buffer was decreased to 6.5 (PIPES) under the standard conditions, inhibition studies with 3′dATP as inhibitor resulted in competetive patterns when isoleucine was the variable substrate, and noncompetitive types were observed when the ATP or $tRNA^{Ile}$ concentration was varied (Table III). These patterns indicate a new type of mechanism that was not observed before in experiments with isoleucyl-tRNA synthetase from yeast. However, similar patterns were obtained with some other ATP analogues and isoleucyl-tRNA synthetase from *E. coli* (Freist et al., 1982) and also with 3′dATP in the misacylation of $tRNA^{Val}$ with isoleucine and isoleucyl-tRNA synthetase from yeast as described below (Tables IV and II). The mechanism to which these inhibition patterns are assigned is the rapid equilibrium sequential random ter-ter mechanism. Thus, the decrease of pH seems to change the ordered sequence of substrate addition into a random order. The $k_{cat}$ value is about half of that observed under standard conditions at pH 7.65; the apparent $K_m$ of $tRNA^{Ile}$ is unchanged.

Table IV: Patterns of Bisubstrate Kinetics and Inhibition by Products and Inhibitors in the Aminoacylation of $tRNA^{Val}$ with Isoleucine and Isoleucyl-tRNA Synthetase under Different Conditions and $K_i$ Values of Inhibitors

| conditions | changing fixed substrate (a), product (b), or inhibitor (c) | variable substrate[a] ATP | Ile | $tRNA^{Val}$ | $K_i$ (mM) |
|---|---|---|---|---|---|
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$ | (a) Ile | r | | | |
| | (a) $tRNA^{Val}$ | r | | | |
| | (b) AMP | c | c | c | 1.1 |
| | (b) $PP_i$ | c | c | c | 0.02 |
| | (c) 3′dATP | nc | c | nc | 0.6 |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$, PPase | (a) Ile | r* | | i | |
| | (a) ATP | | r | i | |
| | (b) AMP | c | c | nc | 1.2 |
| | (c) 3′dATP | c | c | uc | 2.2 |
| | (c) isoleucinol | c | c | uc | 18.1 |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$, EF-Tu–GTP | (a) Ile | r | | | |
| | (a) $tRNA^{Val}$ | r | r | | |
| | (b) AMP | c | c | c | 3.2 |
| | (b) $PP_i$ | c | c | c | 0.02 |
| | (c) 3′dATP | nc | c | nc | 1.3 |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$, EF-Tu–GTP, PPase | (c) 3′dATP | nc | c | nc | 1.7 |
| pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$, EF-Tu–GTP, PPase, spermine | (a) Ile | r | | | |
| | (a) $tRNA^{Val}$ | i | i | | |
| | (b) AMP | uc | uc | nc | 3.8 |
| | (c) 3′dATP | uc | c | uc | 0.4 |
| | (c) isoleucinol | uc | c | uc | 66.7 |
| pH 7.65, 0.1 M KCl, 1 mM $Mg^{2+}$, EF-Tu–GTP, PPase, spermine | (c) 3′dATP | uc | c | uc | 0.3 |
| pH 9.0, 8 mM $Mg^{2+}$ | (a) Ile | r | | | |
| | (a) $tRNA^{Val}$ | i | i | | |
| | (b) AMP | uc | uc | nc | 2.8 |
| | (b) $PP_i$ | nc | nc | c | 0.03,[b] 0.04[c] |
| | (c) 3′dATP | nc | nc | uc | 0.13,[b] 0.11[c] |

[a] c, competitive; nc, noncompetitive; uc, uncompetitive; r, reversibly connected; i, irreversibly connected; r*, reversibly connected, intersecting on the vertical axis. [b] Slope. [c] Intercept.

(2) *Aminoacylation of $tRNA^{Val}$.* The high mischarging capacity of the $tRNA^{Val}$ preparation enabled us to carry out the first initial rate kinetic studies in a misacylation reaction. Bisubstrate kinetics were started with isoleucine as the changing fixed substrate and ATP as the variable substrate; the reciprocal plots intersect at a point to the left of the vertical axis (Table IV). Similar patterns were observed when $tRNA^{Val}$ is the changing fixed substrate and the ATP concentration is varied (Table IV). These observations alone already exclude a ping-pong mechanism and indicate a sequential one. The sequential addition of the three substrates must lead to a ter release of products. Inhibition studies with pyrophosphate and AMP (Table IV) showed competitive inhibition when all three substrates were used as the variable ones. This observation is consistent with a rapid equilibrium random ter-ter mechanism (Fromm, 1975; Segel, 1975b; Table II). Although 3′dATP is a structural analogue of ATP, it showed competitive inhibition with respect to the amino acid as was observed sometimes before for ATP analogues (Freist et al., 1981a,b) and aminoacyl-tRNA synthetases (Table IV). Inhibition with respect to the other two substrates showed

classic noncompetitive patterns and confirmed the rapid equilibrium random ter-ter mechanism (Table IV). The catalytic constant $k_{cat}$ obtained for this misacylation reaction is on average 0.03 $s^{-1}$, a nearly 30-fold lower value than observed for the cognate aminoacylation under the same conditions. The $K_m$ value of the tRNA, however, was nearly unchanged.

A different mechanism was observed when inorganic pyrophosphatase was added to the standard assay. In bisubstrate kinetics, first isoleucine was used as the changing fixed substrate and ATP as the variable substrate. The reciprocal plots intersected on the vertical axis (Table IV), an observation that is typical for mechanisms with rapid equilibrium segments (Segel, 1975c; Fromm, 1975b) and shows a reversible connection of the two enzyme forms to which ATP and isoleucine are added. When isoleucine is the changing fixed substrate and $tRNA^{Val}$ is the variable substrate, parallel plots were observed, indicating the release of products from the enzyme between addition of the two substrates (Table IV). Initial rate kinetics with ATP as changing fixed substrate and isoleucine as variable substrate resulted in reciprocal plots that intersected to the left of the vertical but that on the horizontal axis showed again reversible connection of the enzyme forms to which these two substrates are bound (Table IV). When ATP was the changing fixed substrate and the $tRNA^{Val}$ concentration was varied, again parallel plots were observed, which indicate release of products between addition of the two substrates (Table IV).

Inhibition experiments with isoleucinol as isoleucine analogue caused competitive inhibition types with respect to ATP and isoleucine (Table IV). These results indicate an ordered addition with isoleucine first in a rapid equilibrium mechanism (Segel, 1975c). When $tRNA^{Val}$ is the variable substrate, parallel patterns are observed (Table IV), which show again that products must be released between addition of the small substrates and the tRNA.

3′dATP is also a competitive inhibitor when ATP and isoleucine are the variable substrates (Table VI), and this shows that the compound again acts as would be expected for an analogue of the substrate isoleucine. With respect to $tRNA^{Val}$, uncompetitive patterns are obtained, which once more indicate product release between the small substrates and the tRNA (Table IV). Product inhibition by AMP exhibits competitive types when isoleucine or ATP is the variable substrate (Table IV), which indicate that AMP is released before addition of these two substrates. When the $tRNA^{Val}$ concentration is varied, noncompetitive patterns of the classic type are observed (Table IV). This shows that AMP must be released directly after addition of $tRNA^{Val}$ to the enzyme.

From the results of bisubstrate kinetics and inhibition by substrate analogues and products for the order of substrate addition in the mischarging of $tRNA^{Val}$ with isoleucine by isoleucyl-tRNA synthetase in the presence of pyrophosphatase, a bi-bi uni-uni ping-pong mechanism with a rapid equilibrium segment can be postulated (Table II). In the reaction sequence, isoleucine is the first substrate to be added followed by ATP (rapid equilibrium segment); then, the two products pyrophosphate and Ile-$tRNA^{Val}$ must be released in unknown order, $tRNA^{Val}$ is added, and AMP is released. The $K_m$ value of $tRNA^{Val}$ (0.002 mM) was found to be twice as high as before, whereas $k_{cat}$ is also enhanced to 0.054 $s^{-1}$.

When the misacylation was conducted under the standard conditions in the presence of EF-Tu–GTP complex, similar results as under standard conditions without additives were obtained. Bisubstrate kinetics, product inhibition, and inhibition patterns obtained with 3′dATP indicated the existence of the rapid equilibrium random ter-ter mechanism also in the presence of EF-Tu–GTP complex (Table IV). The $K_m$ of $tRNA^{Val}$ (0.003 mM) and the $k_{cat}$ value of this reaction (0.027 $s^{-1}$) appear very similar to those ones observed in absence of EF-Tu–GTP. The presence of inorganic pyrophosphatase in this assay did not change the results as shown by inhibition patterns observed with 3′dATP (Table IV).

However, a different mechanism was discovered when besides EF-Tu–GTP complex and pyrophosphatase also spermine was present in the reaction mixture. When in bisubstrate kinetics isoleucine was taken as the changing fixed substrate and ATP as the variable substrate, the reciprocal plots intersected to the left of the vertical axis and indicated a reversible connection of the enzyme forms to which the two small substrates are added (Table IV). When $tRNA^{Val}$ was the changing fixed substrate and ATP or isoleucine was the variable substrate, parallel patterns were observed that showed that the enzyme–substrate complexes to which the small substrates and the tRNA are bound are separated by release of one or more products (Table IV).

Inhibition caused by isoleucinol is competitive with respect to isoleucine and uncompetitive when ATP or $tRNA^{Val}$ is the variable substrate (Table IV). Because from bisubstrate kinetics it could be concluded that ATP and isoleucine add to enzyme forms that are reversibly connected, the only explanation for the uncompetitive inhibition type obtained with isoleucinol with respect to ATP is that ATP is added before isoleucine in an ordered sequence. The uncompetitive type observed when the $tRNA^{Val}$ concentration is varied must be caused by product release between addition of the two small substrates and $tRNA^{Val}$ as is also indicated by bisubstrate kinetics. 3′dATP acts in this aminoacylation pathway again like an analogue of isoleucine and causes the same types of inhibition as isoleucinol (Table IV). The product AMP showed uncompetitive inhibition with respect to ATP and isoleucine, indicating that between addition of these substrates and the enzyme form from which AMP dissociates other products must be released (Table IV). When the $tRNA^{Val}$ concentration is varied, inhibition appears to be noncompetitive, which shows that the enzyme–substrate complex to which $tRNA^{Val}$ adds is reversibly connected to that form from which AMP is released (Table IV).

The results of the initial rate kinetic studies are consistent with a bi-uni uni-bi ping-pong mechanism in which ATP is added first followed by isoleucine, then a product (most probably pyrophosphate) is released, $tRNA^{Val}$ is added, and then AMP and isoleucyl-$tRNA^{Val}$ dissociate from the enzyme (Table IV). In this aminoacylation pathway, the $K_m$ of $tRNA^{Val}$ is 0.002 mM and the $k_{cat}$ 0.023 $s^{-1}$, values that are very similar to those observed in the other misacylation pathways.

When the inhibition experiments with 3′dATP in presence of EF-Tu–GTP, pyrophosphatase, and spermine were repeated at the physiological $Mg^{2+}$ concentration, the same inhibition types were observed as in higher $Mg^{2+}$ concentrations (Table IV). This may indicate that order of substrate addition and product release does not depend on the concentration of magnesium ions.

A similar ping-pong mechanism was detected under the "special misacylation conditions". When bisubstrate kinetics were conducted with isoleucine as changing fixed substrate and ATP as variable substrate, the reciprocal plots intersected to the left of the vertical axis, indicating reversible connection of the two enzyme forms to which the two small substrates

are added (Table IV). With $tRNA^{Val}$ as changing fixed substrate and ATP or isoleucine as variable substrate, the reciprocal plots were parallel and showed that after addition of ATP and isoleucine a product must be released before $tRNA^{Val}$ is added (Table IV). 3′dATP as inhibitor caused noncompetitive inhibition with respect to ATP and isoleucine and uncompetitive patterns when the $tRNA^{Val}$ concentration is varied (Table IV). The noncompetitive patterns obtained with respect to ATP indicate a multisite system as already observed by us previously for isoleucyl-tRNA synthetases from yeast and from *E. coli* (Freist et al., 1981b, 1982; Freist & Cramer, 1983). This ATP analogue must be able to add to the enzyme before and after addition of ATP, and the enzyme–substrate complex to which $tRNA^{Val}$ is attached must occur in an ordered reaction sequence before addition of ATP and isoleucine or it must be separated by a product release step from those forms to which ATP and isoleucine are added, a possibility that is already indicated by bisubstrate kinetics.

Product inhibition with AMP resulted in uncompetitive patterns with respect to ATP and isoleucine, showing that release of another product must occur between the enzyme form to which the small substrates are added and the form from which AMP dissociates (Table IV). When $tRNA^{Val}$ was the variable substrate inhibition appears as noncompetitive, indicating reversible connection of the enzyme–substrate complexes to which $tRNA^{Val}$ is added and AMP is released (Table IV). Pyrophosphate was a noncompetitive inhibitor with respect to ATP and isoleucine and a competitive one when the $tRNA^{Val}$ concentration was varied (Table IV). This shows that pyrophosphate is released directly before addition of $tRNA^{Val}$ and that the enzyme forms to which the two small substrates ATP and isoleucine are added are reversibly connected to the form that releases pyrophosphate.

The results obtained with initial rate kinetics under the "special aminoacylation conditions" are consistent with a bi-uni uni-bi ping-pong mechanism (Table IV). ATP and isoleucine are added first in unknown order because no inhibitors were available that showed competitive inhibition with respect to these substrates. After addition of the small substrates, pyrophosphate is released followed by addition of $tRNA^{Val}$; then, AMP and Ile-$tRNA^{Val}$ leave the enzyme. The $K_m$ of $tRNA^{Val}$ in this misacylation pathway is 0.001 mM; the catalytic constant $k_{cat}$ is lowered to 0.005 $s^{-1}$.

(*3*) *Calculation of Discrimination Factors.* For the cognate aminoacylation, four different pathways of the reaction are observed in our experiments. The $k_{cat}$ value for "misacylation conditions" (pH 9, 8 mM $MgSO_4$) is found to be extremely low; for all other conditions, $k_{cat}$ is in the range of 0.1–1.4 $s^{-1}$ (Table II). The $K_m$ of $tRNA^{Ile}$ is varied by a factor of 10. In the misacylation reaction, $k_{cat}$ values are relatively constant between 0.02 and 0.05 $s^{-1}$ except that one observed under the "misacylation conditions", which is again very low. The apparent $K_m$ of $tRNA^{Val}$ is also relatively invariable with values between 0.001 and 0.003 mM (Table II). The overall discrimination factor $D$ can be conveniently calculated from the equation $D = (k_{cat\ tRNA^{Ile}} K_{m\ tRNA^{Val}})/(K_{m\ tRNA^{Ile}} k_{cat\ tRNA^{Val}})$ (Fersht, 1977). The calculated factors show a good conformance with those ones obtained in the mixing experiments (Table II) except that obtained for tRNA concentrations higher than 1 $\mu$M (Table II) when the equation given above may not be valid.

## Discussion

The discrimination between $tRNA^{Val}$ and $tRNA^{Ile}$ preparations from yeast by isoleucyl-tRNA synthetase from the same source was investigated in three different approaches. The results for the overall discrimination factors obtained under different conditions show a good conformance in the three sets of experiments. Naturally, these in vitro experiments can only reveal tendencies that may also occur under in vivo conditions. The reaction conditions chosen in this work may be regarded as a way of stepwise approximation to physiological conditions but are still differing from in vivo occurrences. Compared with the accuracy achieved in the standard assay conditions (pH 7.65, 0.1 M KCl, 15 mM $Mg^{2+}$), addition of pyrophosphatase enhances the discrimination factor whereas the presence of elongation factor Tu–GTP complex has only a small decreasing effect (Tables I and II). However, by further addition of pyrophosphatase, discrimination is diminished to about one-third of the former value. In this reaction mixture containing EF-Tu–GTP complex and pyrophosphatase, spermine does not change the accuracy; however, if the $Mg^{2+}$ concentration is lowered to the physiological value of 1 mM, a slight increase is observed. Changing of the pH to higher or lower values results in the same degree of discrimination as was achieved by addition of pyrophosphatase.

The initial discrimination factors calculated from overall discrimination and proofreading factors are as low as could be expected from the small difference in association constants of $tRNA^{Val}$ and $tRNA^{Ile}$ and isoleucyl-tRNA synthetase (Lam & Schimmel, 1975). The observed factors differ only in a small range under the conditions applied and suggest that the tRNAs are first bound in a relatively unspecific manner in an "initial enzyme–tRNA complex", which after a conformational change may become more specific for its proofreading activity. Two or more step binding processes have already been postulated for some aminoacyl-tRNA synthetases [see, e.g., Krauss et al. (1979) and von der Haar & Cramer (1978)]. It must be considered as a real possibility that under in vivo conditions during the aminoacylation process more effective correction steps occur that result in higher accuracies up to the range of $10^4$–$10^5$ as calculated for misacylations by other authors (Yamane & Hopfield, 1977; Savageau & Freter, 1979). This is all the more likely since it has been found that modified tRNAs like $tRNA^{Ile}$-C-C-3′dA and $tRNA^{Ile}$-C-C-A(3′$NH_2$) can be aminoacylated with valine because a decisive proofreading step is lost. The $tRNA^{Val}$ isoacceptor that is responsible for the high misacylation rates in our experiments can also be regarded as a modified $tRNA^{Ile}$ that perhaps diminishes the effect of the correction mechanism (W. Freist and F. Cramer, unpublished results).

Because in our experiments the aminoacylation rates are higher than observed rates of hydrolysis of isoleucyl-tRNA synthetase–isoleucyl adenylate complexes (Fersht, 1979) and the observed proofreading factors are consistent with the different overall discrimination factors, it can be assumed that proofreading occurs in the misacylation of $tRNA^{Val}$ with isoleucine and isoleucyl-tRNA synthetase. Generally, it can be stated that proofreading can be used by aminoacyl-tRNA synthetases to reject tRNAs that are normally specific for other amino acids, but not all synthetases may act with such mechanisms. For isoleucyl-tRNA synthetase, a proofreading process seems to be necessary because $tRNA^{Val}$ and $tRNA^{Ile}$ are perhaps very similar in their recognition sites.

The different orders of substrate addition and product release observed by initial rate kinetic experiments show that aminoacylation of $tRNA^{Ile}$ can be achieved by many different pathways, which include sequential and ping-pong, steady-state, and rapid equilibrium mechanisms. In every case, the chemistry of aminoacylation may be the same whereas the applied changes of assay conditions may be responsible for

minor conformational changes of the enzyme that result in different order of substrate additions and product release. The misacylation of $tRNA^{Val}$ with isoleucine catalyzed by isoleucyl-tRNA synthetase is the first misacylation reaction investigated by initial rate kinetics. In every case, substitution of the cognate $tRNA^{Ile}$ by $tRNA^{Val}$ results in a change of order of substrate addition and product release and, in this way, of the whole catalytic process. This observation must be taken into account for detailed calculations of error rates if rate constants of single reaction steps are used in network models. No strong correlation between reaction pathway and accuracy can be observed; the discrimination factors observed within one pathway are also considerably variable.

The listed $k_{cat}$ and $K_m$ values (Table II) show clearly that the variability of the overall discrimination is most dependent on the variations of the kinetic constants observed for the cognate aminoacylation. A possible explanation for the differences in cognate aminoacylation might be that the enzyme may act under different conditions with one or two molecules of tRNA attached to it. Models with two tRNA molecules complexed to the enzymes have been postulated by some authors [e.g., Thiebe (1978), Kern & Gangloff (1981), and Freist et al. (1982)]. The invariability of the kinetic constants in the misacylation reaction may be observed because this reaction may be only possible if one tRNA molecule is complexed by the enzyme. A parallel observation has been made with phenylalanyl-tRNA synthetase from yeast, which binds two molecules of cognate tRNA and only one of noncognate species (Krauss et al., 1976).

The discrimination rates observed in our experiments vary by a factor of about 10, and it does not seem unrealistic to us that such a factor may also occur in the whole translation system working under physiological conditions. Because the error level of misacylation occurring in vivo is estimated to be in the range of 1 in $10^5$ (Yamane & Hopfield, 1977), this factor could vary the error rates between 1 in $10^4$ and 1 in $10^5$, which may be an important order of magnitude.

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## Supplementary Material Available

Six figures containing data displayed in 71 Lineweaver–Burk plots (6 pages). Ordering information is given on any current masthead page.

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# 5S RNA Structure and Interaction with Transcription Factor A. 1. Ribonuclease Probe of the Structure of 5S RNA from *Xenopus laevis* Oocytes†

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ABSTRACT: The structure of *Xenopus laevis* oocyte (Xlo) 5S ribosomal RNA has been probed with single-strand-specific ribonucleases $T_1$, $T_2$, and A with double-strand-specific ribonuclease $V_1$ from cobra venom. The digestion of 5′- or 3′-labeled renatured 5S RNA samples followed by gel purification of the digested samples allowed the determination of primary cleavage sites. Results of these ribonuclease digestions provide support for the generalized 5S RNA secondary structural model derived from comparative sequence analysis. However, three putative single-stranded regions of the molecule exhibited unexpected $V_1$ cuts, found at $C_{36}$, $U_{73}$, $U_{76}$, and $U_{102}$. These $V_1$ cuts reflect additional secondary structural features of the RNA including A·G base pairs and support the extended base pairing in the stem containing helices IV and V which was proposed by Stahl et al. [Stahl, D. A., Luehrsen, K. R., Woese, C. R., & Pace, N. R. (1981) *Nucleic Acids Res. 9*, 6129-6137]. A conserved structure for helix V having a common unpaired uracil residue at Xlo position 84 is proposed for all eukaryotic 5S RNAs. Our results are compared with nuclease probes of other 5S RNAs.

The 5S RNA is found complexed with specific proteins in the ribosomes of all organisms and also exists in 7S ribonucleoprotein (RNP) particles of amphibians, teleosts (Picard et al., 1979), and HeLa cells (Gruissem & Seifart, 1982). This RNA has been shown to be phylogenetically conserved not only in overall length (about 120 nucleotides) but also in sequence at certain nucleotide positions and in the internal chain lengths between conserved residues (Delihas & Andersen, 1982). Secondary structural models for both eukaryotic and eubacterial 5S RNAs have been proposed on the basis of comparative sequence analysis (Nishikawa & Takemura, 1974; Fox & Woese, 1975; Luehrsen & Fox, 1981; Studnicka et al., 1981; Böhm et al., 1981). Recently, these models have merged into one generalized secondary structural model for all phylogenetic classes of 5S RNA (Delihas & Andersen, 1982; Böhm et al., 1982; DeWachter et al., 1982; Kuntzel et al., 1983).

As a part of a study of 5S RNA conformation and RNA-protein interactions, we have probed the 5S RNA from *Xenopus laevis* oocytes (Xlo) with ribonucleases. This information also provides a background for the study of the oocyte

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