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Isoleucyl Transfer Ribonucleic Acid Synthetase. Steady-State Kinetic Analysis†

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ABSTRACT: A steady-state kinetic analysis was conducted of the overall aminoacylation reaction catalyzed by isoleucyl-tRNA synthetase. The patterns of Lineweaver–Burk plots obtained indicated that tRNA adds to the enzyme only after isoleucyl adenylate formation and pyrophosphate release. These kinetic patterns were consistent with the bi-uni-uni-bi Ping Pong mechanism generally accepted for this aminoacyl-tRNA synthetase, but they could also be accommodated by a mechanism in which a second molecule of L-isoleucine added to the enzyme between isoleucyl adenylate formation and aminoacylation of tRNA [Fersht, A.R., & Kaethner, M. M. (1976) *Biochemistry 15*, 818]. The values of the kinetic parameters favor the latter mechanism. The results of this kinetic analysis indicated that the affinity of isoleucyl-tRNA synthetase for Mg·ATP was enhanced upon binding of L-isoleucine and vice versa. It also indicated that the affinity of the enzyme for L-isoleucine is decreased upon binding tRNA and vice versa. The values of dissociation constants calculated for each of the substrates by this study generally compared well with those determined by other authors using a variety of kinetic and equilibrium methods.

The aminoacyl-tRNA synthetases are a group of enzymes which catalyze the esterification of amino acids to their cognate tRNAs in a process of critical importance to protein biosynthesis. The fidelity of translation of genetic information into protein is completely dependent on the specificities of these enzymes. The mechanisms used by these enzymes to ensure faithful esterification of amino acids to the proper tRNAs have therefore been the focus of many years of intensive study [for review articles see Schimmel (1973), Söll & Schimmel (1974), and Kisselev & Favorova (1974)].

The mechanism of the reaction catalyzed by isoleucyl-tRNA synthetase of *Escherichia coli* (EC 6.1.1.5) has been the subject of numerous studies; however, very few of these have attempted to study the overall reaction, as shown in eq 1.

$$\text{Ile} + \text{ATP} + \text{tRNA} \rightarrow \text{Ile-tRNA} + \text{AMP} + \text{PP}_i \quad (1)$$

Steady-state kinetic analysis of the overall reaction has been published in a preliminary, qualitative form (Takeda & Matsuzaki, 1974), but most of our knowledge of the overall reaction mechanism has come from studies of partial reactions. The generally accepted mechanism (Schimmel, 1973; Söll & Schimmel, 1974; Kisselev & Favorova, 1974) holds that isoleucine is first condensed with ATP to form the activated enzyme-bound intermediate, isoleucyl-AMP, followed by transfer of the amino acid to its cognate tRNA, as shown in eq 2. This reaction mechanism, in the terminology of Cleland

$$\begin{gathered}\text{E} + \text{Ile} + \text{ATP} \rightleftharpoons \text{E·Ile-AMP} + \text{PP}_i \\ \text{E·Ile-AMP} + \text{tRNA} \rightleftharpoons \text{Ile-tRNA} + \text{AMP} + \text{E} \quad (2)\end{gathered}$$

(1963a), is bi-uni-uni-bi Ping Pong. On the basis of their studies of partial reactions, Fersht & Kaethner (1976) modified this mechanism. In order to reconcile kinetic constants of various partial reactions, they proposed the addition of a second molecule of isoleucine to the enzyme after the activation step but before the formation of isoleucyl-tRNA, as shown in eq 3a–c.

$$\text{E·tRNA·Ile·ATP} \rightleftharpoons \text{E·tRNA·Ile-AMP} + \text{PP}_i \quad (3a)$$

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$$E\cdot tRNA\cdot Ile\text{-}AMP + Ile \rightleftharpoons E\cdot tRNA\cdot Ile\text{-}AMP\cdot Ile \quad (3b)$$

$$E\cdot tRNA\cdot Ile\text{-}AMP\cdot Ile \rightleftharpoons E + Ile\text{-}tRNA + AMP + Ile \quad (3c)$$

The purposes of our study were to determine by a steady-state kinetic analysis the mechanism of catalysis by isoleucyl-tRNA synthetase and to determine the equilibrium and kinetic constants describing the overall aminoacylation reaction.

Materials and Methods

Purification of Isoleucyl-tRNA Synthetase. Homogeneous isoleucyl-tRNA synthetase from *E. coli* was prepared either by affinity chromatography on blue dextran–Sepharose (Moe & Piszkiewicz, 1976) or by the method of Eldred & Schimmel (1972a). The enzyme obtained from the latter procedure was usually subjected to a final purification step on blue dextran–Sepharose. Purity of the enzyme was checked by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Weber & Osborn, 1969). Protein concentration was determined by the carboxylation modification of the Lowry assay (Ross & Schatz, 1973) and also by using the known extinction coefficient of the enzyme, $\epsilon_{280} = 1.91 \times 10^5$ M^{-1} cm^{-1} (Iaccarino & Berg, 1969).

Kinetics of Isoleucylation of tRNA. Isoleucyl-tRNA synthetase was routinely assayed by the esterification of [^{3}H]isoleucine (ICN) to unfractionated *E. coli* B tRNA (Schwarz/Mann) in 0.05 M Tris-HCl, pH 7.60, at 30 °C according to the method of Muench & Berg (1966). Kinetic measurements of the aminoacylation of unfractionated tRNA by isoleucyl-tRNA synthetase were made at 20 °C in 5 mM sodium phosphate buffer at pH 6.40 with 10 mM 2-mercaptoethanol. Constant components in the reaction mixture were isoleucyl-tRNA synthetase (1.5 μg/mL), inorganic pyrophosphatase (1 unit/mL), and magnesium acetate (5 mM excess over ATP). The concentration of [^{3}H]-L-isoleucine (either 50 or 100 mCi/mmol; ICN) was 100 μM when Mg·ATP or tRNA was the varied substrate, and it was varied from 5 to 100 μM when it was the varied substrate. The concentration of Mg·ATP (Calbiochem) was 1.0 mM when [^{3}H]-L-isoleucine or tRNA was the varied substrate, and it was varied from 5 to 500 μM when it was the varied substrate. The concentration of the Mg·ATP stock solution was determined from the molar extinction coefficient of ATP: ϵ_{259} = 15 400 (Bock et al., 1956). Unfractionated tRNA from *E. coli* B (Schwarz/Mann) was determined to be 0.84% $tRNA^{Ile}$ by weight on the basis of its maximum level of esterification by [^{3}H]-L-isoleucine. Its concentration was 3.0 mg/mL (810 nM $tRNA^{Ile}$) when [^{3}H]-L-isoleucine and Mg·ATP were the varied substrates, and it was varied from 0.20 to 3.00 mg/mL (54 to 810 nM $tRNA^{Ile}$) when it was the varied substrate. At a minimum of six timed intervals, 200-μL aliquots of the reaction mixture were withdrawn and immediately dispensed into cold 5% trichloroacetic acid. After 30 min on ice, the samples were collected on glass fiber filters (Reeve Angel 934AH), rinsed extensively with 5% trichloroacetic acid, given a final rinse of 10 mL of 95% ethanol, dried, and counted by liquid scintillation in toluene–PPO–POPOP. Initial rates were calculated by utilizing the linear regression program of a Texas Instruments SR-51 calculator.

Inorganic Pyrophosphatase Assay. Esterification of isoleucine to tRNA was done in the presence of yeast inorganic pyrophosphatase (pyrophosphate phosphohydrolase; EC 3.6.1.1); the activity of the preparation, purchased from Sigma, was checked at 25 °C in 20 mM Tris-HCl at pH 7.20 (measured at room temperature) containing 1.5 mM sodium pyrophosphate and 1.5 mM magnesium acetate. The inorganic orthophosphate released by the reaction was determined by the method of Baginski et al. (1967). A unit of inorganic pyrophosphatase was defined as that amount of enzyme which will hydrolyze 1 μmol of inorganic pyrophosphate in 1 min under these conditions (Worthington, 1972).

Initial Rate of Formation of Isoleucyl Adenylate. The rate-limiting step of the L-isoleucine-dependent ATP–PP_i exchange reaction catalyzed by isoleucyl-tRNA synthetase is the formation of isoleucyl adenylate (Holler & Calvin, 1972). The rate of ATP–PP_i exchange was determined at 20 °C in 5 mM sodium phosphate at pH 6.40 with 10 mM 2-mercaptoethanol. The reaction mixture contained 100 μM L-isoleucine, 2 mM Mg·ATP, 2 mM Na[^{32}P]PP_i (2.4 mCi/mmol; NEN), 3.0 mg/mL unfractionated tRNA, and magnesium acetate at a concentration 5 mM in excess of the concentration of ATP. The reaction was initiated by the addition of isoleucyl-tRNA synthetase. The final concentration of the enzyme was 1.5 μg/mL. Aliquots (200 μL) were removed at 1-min intervals and dispensed into test tubes containing 50 μL of 14% perchloric acid. Norit (0.3 mL; 12%) was dispensed into each sample. The Norit was then collected on glass fiber filters, washed with 30 mL of 1% $NaPP_i$, dried, and counted by liquid scintillation in toluene–PPO–POPOP. The initial rate was calculated by utilizing the linear regression program of a Texas Instruments SR-51 calculator.

Results

The scheme for the reaction catalyzed by isoleucyl-tRNA synthetase proposed by Fersht & Kaethner (1976) is presented by using the format of King & Altman (1956) in Figure 1. Since the proposed reaction scheme does not specify the order of substrate addition, the figure indicates the addition of tRNA either before or after isoleucyl adenylate formation.

Determination of the order actually used by isoleucyl-tRNA synthetase in binding to its substrates was the first objective of this study. The rate of the overall aminoacylation reaction was determined as a function of the concentration of L-isoleucine, Mg·ATP, and tRNA. For any experiment, the concentrations of two of the substrates were varied while the concentration of the third was held constant. When the data obtained in this manner was plotted by using the method of Lineweaver & Burk (1934), the patterns formed by the lines fitting the experimental points gave qualitative information about the enzyme forms which bind the varied substrates (Cleland, 1963b).

The Lineweaver–Burk plot of the reciprocal of initial velocity vs. the reciprocal of Mg·ATP concentration with L-isoleucine as the changing fixed substrate and tRNA held constant (Figure 2) showed intersecting lines. A similar plot with Mg·ATP as the varied substrate, L-isoleucine the changing fixed substrate, and tRNA held constant (figure not shown) also gave lines which intersected above the 1/substrate axis. This indicated that L-isoleucine and Mg·ATP formed a ternary complex with the enzyme. The fact that the lines intersected above the 1/substrate axes indicated that the affinity of the enzyme for Mg·ATP was enhanced upon binding L-isoleucine and vice versa.

When the concentration of Mg·ATP was held constant, L-isoleucine was the varied substrate, and tRNA was the changing fixed substrate (Figure 3), the Lineweaver–Burk plot showed intersecting lines. Similarly, with Mg·ATP constant, L-isoleucine as the varied substrate, and tRNA as the changing fixed substrate (figure not shown), the plots gave intersecting lines. Thus, L-isoleucine and tRNA formed a ternary complex with the enzyme. The point of intersection was below the 1/substrate axis. This indicated that the affinity of the enzyme

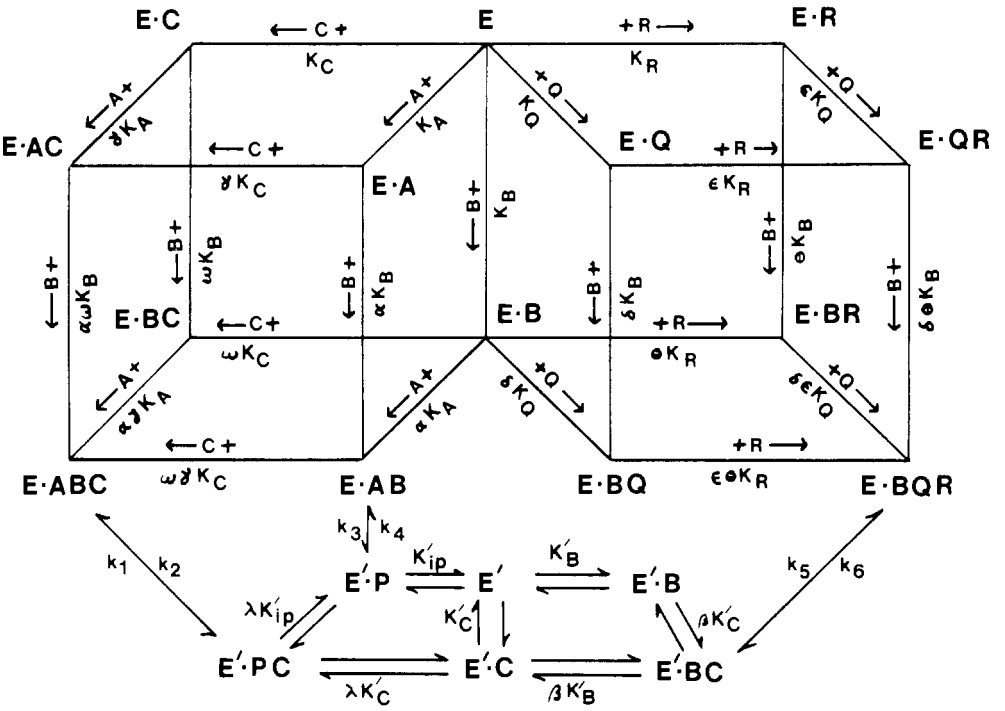


FIGURE 1: King–Altman representation of the reaction scheme proposed for isoleucyl-tRNA synthetase by Fersht & Kaethner (1976). The scheme as written incorporates the assumption that the binding and release of substrates and products is rapid relative to the making and breaking of covalent bonds. The following symbols are used: E = isoleucyl-tRNA synthetase; E′ = E·Ile-AMP; A = Mg·ATP; B = L-isoleucine; C = $tRNA^{Ile}$; P = $Mg{\cdot}PP_i$; Q = AMP; R = Ile-$tRNA^{Ile}$. The dissociation constants are designated K_x, where x is the symbol used for the substrate or product. α, β, γ, δ, ϵ, λ, θ, and ω are interaction factors reflecting the change in the dissociation constant for one ligand upon binding another. The rate constants describe the following reactions: k_1 and k_2, pyrophosphate exchange in the presence of tRNA; k_3 and k_4, pyrophosphate exchange in the absence of tRNA; k_5 and k_6, transfer of isoleucyl moiety from AMP to tRNA.

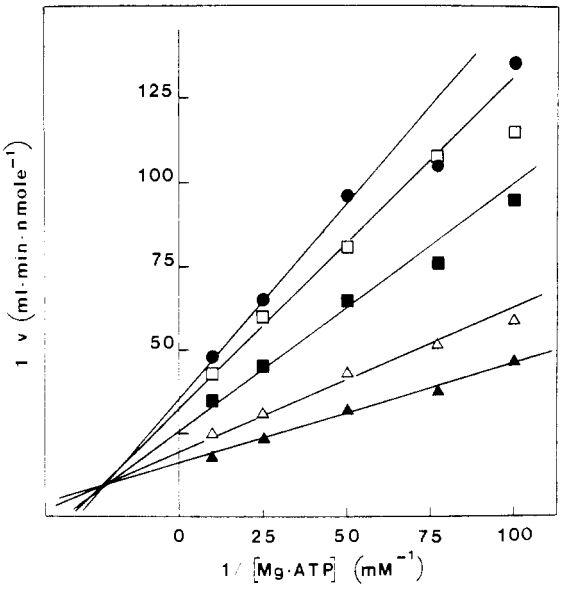


FIGURE 2: Kinetics of aminoacylation at 20 °C of unfractionated tRNA as a function of Mg·ATP and L-isoleucine concentrations. Mg·ATP was the varied substrate with L-isoleucine as the changing fixed substrate. Concentrations of L-isoleucine used were 10 (●), 13 (□), 20 (■), 40 (△), and 100 μM (▲). Conditions and procedures used were those described under Materials and Methods.

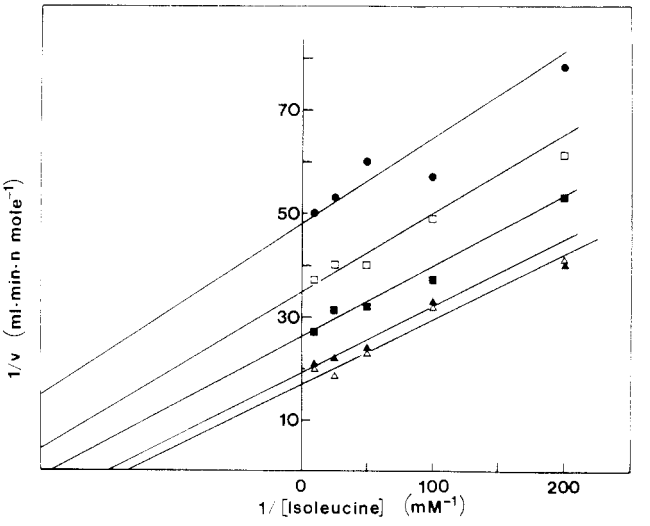


FIGURE 3: Kinetics of aminoacylation at 20 °C of unfractionated tRNA as a function of L-isoleucine and tRNA concentrations. L-Isoleucine was the varied substrate and tRNA was the changing fixed substrate. Concentrations of tRNA used were 0.20 (●), 0.33 (□), 0.50 (■), 1.00 (▲), and 2.00 mg/mL (△). Conditions and procedures used were those described under Materials and Methods.

for L-isoleucine is decreased upon binding tRNA and vice versa.

When the concentration of L-isoleucine was held constant, tRNA was the varied substrate, and Mg·ATP was the changing fixed substrate (Figure 4), the Lineweaver–Burk plots showed parallel lines. Also with L-isoleucine constant, tRNA as the varied substrate, and Mg·ATP as the changing fixed substrate, the plots gave parallel lines (figure not shown). This indicated that the binding of Mg·ATP to the enzyme is separated by an irreversible step (i.e., release of pyrophosphate) from the binding of tRNA.

Thus, during the aminoacylation of tRNA by isoleucyl-tRNA synthetase, the enzyme can exist in a form which simultaneously binds L-isoleucine and Mg·ATP. It can also exist in a form which binds both L-isoleucine and tRNA. It does not exist in a form which simultaneously binds Mg·ATP and tRNA. These observations are consistent with the reaction scheme of Fersht & Kaethner (1976) if, during the overall aminoacylation reaction, tRNA binds to the enzyme only after the formation of isoleucyl adenylate. This simplified reaction

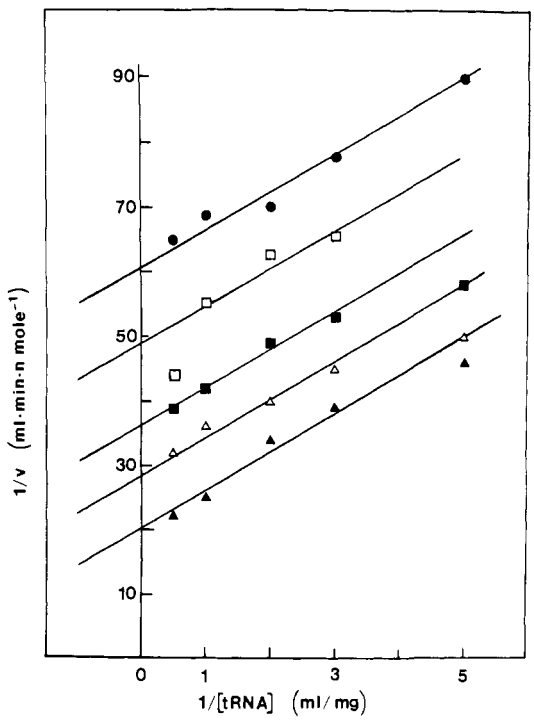


FIGURE 4: Kinetics of aminoacylation at 20 °C of unfractionated tRNA as a function of tRNA and Mg·ATP concentrations. tRNA was the varied substrate and Mg·ATP was the changing fixed substrate. Concentrations of Mg·ATP used were 5 (●), 10 (□), 20 (■), 50 (△), and 500 μM (▲). Conditions and procedures used were those described under Materials and Methods.

scheme is presented in Figure 5.

A kinetic rate equation describing this mechanism has been derived (Moe, 1978). The initial rate of the aminoacylation reaction as presented in Figure 5 is given by eq 4, where each

$$\frac{v}{V_{max}} = ([A][B][C]) \Bigg/ \left[\beta K_{ib}' K_c' \left(\frac{[B]}{K_{ib}'} + \frac{[C]}{K_c'} + \frac{[B][C]}{\beta K_{ib}' K_c'} + 1 \right)[A] + \frac{k_3}{k_1} \alpha K_A K_B [C] \left(\frac{[A]}{K_A} + \frac{[B]}{K_B} + \frac{[A][B]}{K_A K_B} \right) \right] \quad (4)$$

of the constants and symbols is as defined in the legend of Figure 1. Complex kinetic constants were extracted from secondary plots of the slope and intercept values of the primary plots (Figures 2–4). For example, in Figure 2 a Lineweaver–Burk plot is shown in which Mg·ATP (A) is the varied substrate and L-isoleucine (B) is the changing fixed substrate. The slope of this line is given by eq 5

$$\text{slope} = \frac{1}{V_{max}} \frac{k_3}{k_1} \alpha \left(K_A \frac{K_B}{[B]} + 1 \right) \quad (5)$$

(Moe, 1978). According to eq 5, one may plot the slopes of the lines in Figure 2 vs. 1/[L-isoleucine] (i.e., 1/[B]). The intercept of this secondary plot (not shown) is $(1/V_{max})(k_3/k_1)\alpha K_A$; the slope is $(1/V_{max})(k_3/k_1)\alpha K_A K_B$. The ratio of the slope and the intercept of the secondary plot is K_B. In a similar manner secondary plots of the data in Figures 2–4 were generated, and complex kinetic constants were derived (figures not shown).

The rate constant (k_1) of enzyme-catalyzed formation of isoleucyl adenylate was determined by measuring the initial rate of pyrophosphate exchange in the presence of tRNA (figure not shown).

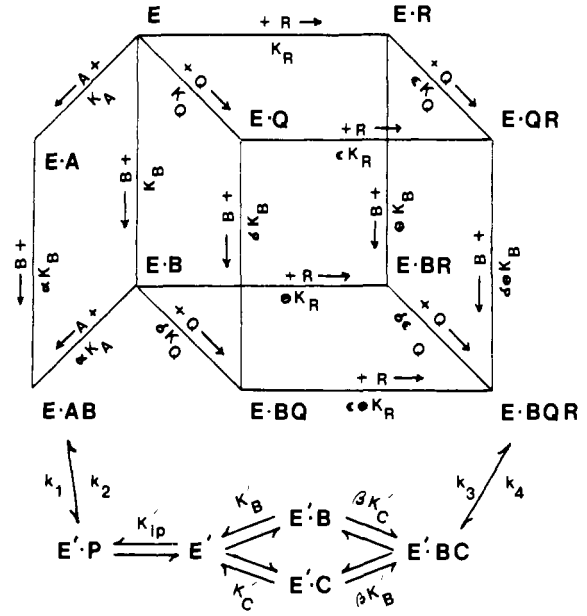


FIGURE 5: King–Altman representation of the Fersht & Kaethner (1976) reaction scheme for isoleucyl-tRNA synthetase as modified by this study. In this representation, tRNA adds to the enzyme only after formation of isoleucyl adenylate and release of pyrophosphate. All symbols and constants are as defined in the legend of Figure 1.

Table I: Kinetic and Equilibrium Constants for Isoleucyl-tRNA Synthetase

K_A[a] = K_{MgATP}	2.6×10^{-3} M
$K_B = K_{Ile}$	5.9×10^{-5} M
$K'_{ib} = K'_{Ile}$	1.4×10^{-6} M
$K'_c = K'_{tRNA}$	1.6×10^{-8} M
V_{max}	8.3×10^{-8} M/min
k_3/k_1	7.9×10^{-3}
β	9.3

[a] The results of this study yield only a qualitative value for $0 < \alpha < 1$. K_A was calculated by using the value determined for α under equilibrium conditions. This was $\alpha = 0.7$ (Holler et al., 1975).

A summary of the rate and equilibrium constants obtained in this analysis is presented in Table I. A summary of the values obtained from the secondary plots is presented in Table II. This table includes the definition of these values in terms of kinetic and equilibrium constants and presents both the experimental values and the theoretical values calculated by using the constants derived in this study.

Discussion

This report describes a steady-state kinetic analysis of the reaction catalyzed by isoleucyl-tRNA synthetase of *E. coli*. Initial velocity of the overall aminoacylation reaction was determined as a function of substrate concentration and analyzed in the qualitative manner described by Cleland (1963b). These kinetic patterns were qualitatively in agreement with the bi-uni-uni-bi Ping Pong mechanism generally accepted for this aminoacyl-tRNA synthetase. They could also be accommodated by a mechanism first proposed by Fersht & Kaethner (1976) on the basis of their analysis of partial reactions in which a second molecule of L-isoleucine added to the enzyme between isoleucyl adenylate formation and aminoacylation of tRNA. In view of our (Moe & Piszkiewicz, 1979) results which indicate that inhibition patterns obtained in the presence of blue dextran are not consistent with a bi-uni-uni-bi Ping Pong mechanism but are consistent with the addition of a second L-isoleucine, the latter mechanism is preferred. A rate law based on this mechanism

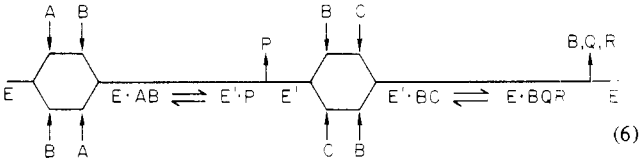
Table II: Summary of Experimentally Derived Values for Equations Relating Kinetic and Equilibrium Constants for Isoleucyl-tRNA Synthetase

expression	source[a]	exptl	calcd[b]
$\frac{1}{V_{max}}\frac{k_3}{k_1}\alpha K_A K_B$	slope of replot of slopes from Figure 2	1.1×10^{-2} M min	1.0×10^{-2} M min
	slope of replot of slopes from plots of $1/v$ vs. 1/[Ile] with Mg·ATP as the changing fixed substrate and tRNA constant	1.0×10^{-2} M min	
$\frac{1}{V_{max}}\frac{k_3}{k_1}\alpha K_A$	intercept of replot of slopes from Figure 2	1.7×10^2 min	1.7×10^2 min
	slope of replot of intercepts from plots of $1/v$ vs. 1/[Ile] with Mg·ATP as changing fixed substrate and tRNA constant	1.7×10^2 min	
$\frac{1}{V_{max}}\left(\frac{k_3}{k_1}+\frac{\beta K'_c}{[C]}+1\right)$	intercept of replot of intercepts from Figure 2	1.5×10^7 min M^{-1}	1.4×10^7 min M^{-1}
	intercept of replot of intercepts from plot of $1/v$ vs. 1/[Ile] with Mg·ATP as changing fixed substrate and tRNA constant	1.5×10^7 min M^{-1}	
$\frac{1}{V_{max}}\left[\frac{k_3}{k_1}\alpha K_B+\beta K'_{ib}\left(\frac{1+K'_c}{[C]}\right)\right]$	slope of replot of intercepts from Figure 2	1.7×10^2 min	1.6×10^2 min
	intercept of replots of slopes from plot of $1/v$ vs. 1/[Ile] with Mg·ATP as the changing fixed substrate and tRNA constant	1.7×10^2 min	
$\frac{1}{V_{max}}\left(\frac{K_B}{[B]}+1\right)\alpha K_A$	slope of plot of $1/v$ vs. 1/[Mg·ATP] at constant tRNA	3.0×10^2 min	2.7×10^2 min
	slope of replot of intercepts from Figure 4	3.0×10^2 min	
$\frac{1}{V_{max}}\beta K'_c\left(\frac{K'_{ib}}{[B]}+1\right)$	slope of replot of slopes from plot of $1/v$ vs. 1/[Mg·ATP] at constant tRNA	1.8 min	1.8 min
	slope of Figure 4	1.5 min	
$\frac{1}{V_{max}}\left[\frac{k_3}{k_1}\left(\frac{\alpha K_B}{[B]}+1\right)+\frac{\beta K'_{ib}}{[B]}+1\right]$	intercept of replot of slopes from plot of $1/v$ vs. 1/[Mg·ATP] at constant tRNA	2.0×10^7 min M^{-1}	1.4×10^7 min M^{-1}
	intercept of replot of intercepts from Figure 4	2.0×10^7 min M^{-1}	
$\frac{1}{V_{max}}\beta K'_{ib}K'_c$	slope of replot of slopes from Figure 3	2.7×10^{-6} M min	2.5×10^{-6} M min
	slope of replot of slopes from plot of $1/v$ vs. 1/[tRNA] with Ile as the changing fixed substrate and Mg·ATP constant	2.5×10^{-6} M min	
$\frac{1}{V_{max}}\beta K'_c$	slope of replot of intercepts from Figure 3	1.8 min	1.8 min
	intercept of replot of slopes from plot of $1/v$ vs. 1/[tRNA] with Ile as the changing fixed substrate and Mg·ATP constant	1.8 min	
$\frac{1}{V_{max}}\left[\frac{k_3}{k_1}\left(\frac{\alpha K_A}{[A]}+1\right)+1\right]$	intercept of replot of intercepts from Figure 3	1.3×10^7 min M^{-1}	1.2×10^7 min M^{-1}
	intercept of replot of intercepts from plots of $1/v$ vs. 1/[tRNA] with Ile as the changing fixed substrate and Mg·ATP constant	1.3×10^7 min M^{-1}	
$\frac{1}{V_{max}}\left[\frac{k_3}{k_1}\alpha K_B\left(\frac{K_A}{[A]}+1\right)+\beta K'_{ib}\right]$	intercept of replot of slopes from Figure 3	1.2×10^2 min	1.7×10^2 min
	slope of replot of slopes from plot of $1/v$ vs. 1/[tRNA] with Ile as the changing fixed substrate and Mg·ATP constant	1.5×10^2 min	

[a] Replots are secondary plots of slopes or intercepts of the primary Lineweaver-Burk plots (Figures 2-4) vs. the reciprocal of the concentrations of the changing fixed substrates. [b] By use of values from Table I.

(Figure 5) was derived [eq 4; also see Moe (1978)], and the values of the various equilibrium and kinetic constants describing the reaction were determined.

The most innovative feature of the reaction scheme proposed by Fersht & Kaethner (1976) is the addition of a second molecule of L-isoleucine between the formation of isoleucyl-AMP and the transfer of the isoleucyl moiety from the adenylate to tRNA. The results of this study are consistent with this novel mechanism. However, Fersht & Kaethner (1976) did not address themselves to defining the order of addition of substrates to the enzyme. The results of this study indicate that tRNA adds only after the formation of isoleucyl adenylate and the release of inorganic pyrophosphate. Thus, the reaction used by isoleucyl-tRNA synthetase in the aminoacylation of tRNA is represented (Cleland, 1963a) by eq 6



(6)

where the symbols are as defined in the legend of Figure 1. The order or product release after the formation of E·BQR cannot be determined from this study of initial rate. The values of dissociation constants calculated for each of the substrates by this study generally compared well with those determined by other authors using a variety of kinetic and equilibrium methods (Table III).

Table III: Dissociation Constants for Substrates of Isoleucyl-tRNA Synthetase

$\alpha K_{Mg\cdot ATP}$ (mM)	1.8[a]
	1.2[b]
	0.95[c]
αK_{Ile} (μM)	40[a]
	18[c]
	6[b]
K'_{Ile} (μM)	1.4[a]
	6.8[b]
	7.7-11[d]
$\beta K'_{Ile}$ (μM)	13[a]
	25[d]
	5.7[b]
K'_{tRNA} (M)	1.6×10^{-8} [a]
	4×10^{-8} [e]
β	9.3[a]
	4-5[d]

[a] This study; pH 6.40; 5 mM Mg^{2+}; 20 °C. [b] Fersht & Kaethner (1976); pH 7.78; 10 mM Mg^{2+}; 25 °C. [c] McNeil & Schimmel (1972); pH 6.0; 10 mM Mg^{2+}; 17 °C. [d] Hustedt et al. (1977); pH 7.5; 25 °C. [e] Lam & Schimmel (1975); pH 6.5; 5 mM Mg^{2+}; 17 °C.

The nature of the participation of the second molecule of L-isoleucine is unclear. The results of this study are consistent with the addition of a second L-isoleucine after isoleucyl adenylate formation to either the E·Ile-AMP or the E·Ile-AMP·tRNA form of the enzyme. Since it adds before the formation of isoleucyl-tRNA, the second L-isoleucine cannot simply act to competitively displace the isoleucyl moiety from the amino acid binding site after isoleucyl-tRNA formation. However, it could serve to decrease the affinity between the first isoleucyl moiety and the L-isoleucine binding site after isoleucyl adenylate formation. If this indeed occurs, the isoleucyl adenylate would still remain enzyme bound but presumably would be rendered more reactive to tRNA. Attempts to demonstrate by equilibrium methods the simultaneous binding to isoleucyl-tRNA synthetase of two molecules of L-isoleucine (Fersht & Kaethner, 1976; Hustedt et al., 1977) or L-isoleucine and isoleucyl adenylate (Mulvey & Fersht, 1977) have been unsuccessful. This indicates that if an enzyme form binding both L-isoleucine and isoleucyl adenylate exists, it must be unstable relative to the enzyme·isoleucyl adenylate complex. This would be consistent with a role of the unstable complex in a step toward the transition state leading to aminoacylation.

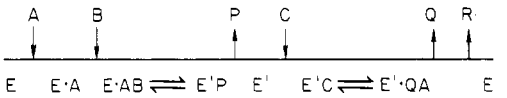
The possibility that the second molecule of L-isoleucine acts to destabilize the enzyme·isoleucyl adenylate complex is also suggested by two studies using analogues of isoleucyl-AMP. Norvalinyl adenylate binds to isoleucyl-tRNA synthetase with a dissociation constant of 5.1×10^{-7} M (Flossdorf et al., 1977). While saturating concentrations of ATP cannot displace this ester, saturating concentrations of L-isoleucine completely displace it from the enzyme. Similarly, the addition of L-isoleucine causes the immediate expulsion of isoleucyl-tRNA synthetase bound valyl-AMP (Fersht, 1977). Thus, the destabilization of the aminoacyl adenylate complex upon binding L-isoleucine could serve as a point of discrimination against activated noncognate amino acid.

The data gathered in this study indicate that tRNA can add either to the enzyme·Ile-AMP complex or to the enzyme·Ile-AMP·Ile complex (eq 6). If tRNA adds before the second L-isoleucine, the transfer of the isoleucyl moiety from AMP to tRNA does not occur until after the second L-isoleucine is bound. Thus, the effect of the added tRNA alone on the reactivity of the isoleucyl adenylate is difficult to assess. For the enzyme·valyl-AMP complex, addition of $tRNA^{Ile}$ causes rapid hydrolysis of valyl-AMP (Fersht, 1977). This may indicate that bound $tRNA^{Ile}$ serves to increase the reactivity of the enzyme-bound aminoacyl adenylate. For isoleucyl-AMP, this would presumably enhance the rate at which the isoleucyl moiety is transferred from AMP to tRNA. For valyl-AMP, the increased reactivity leads to hydrolysis.

The observation that the binding of L-isoleucine causes a decrease in the affinity of the enzyme for tRNA has now been made by equilibrium (Hustedt et al., 1977) and kinetic (this study) means. The decreased affinity of the enzyme for $tRNA^{Ile}$ upon binding L-isoleucine may reflect a more rigorous selection against noncognate determinants by the enzyme·L-isoleucine complex. This may be analogous to the observation that the weak deacylation activity of the enzyme toward isoleucyl-$tRNA^{Ile}$ (Schrier & Schimmel, 1972) functions as a powerful error-correcting activity against valyl-$tRNA^{Ile}$ (Eldred & Schimmel, 1972b).

There is no reason to assume that the mechanism of aminoacylation of tRNA is identical for all synthetases. Though the chemistry of bond making and bond breaking is likely to be similar for all the synthetases, the relative rates of the individual processes may differ and hence lead to different observed mechanisms. The added complication that some synthetases possess multiple, interacting binding sites for tRNA (Krauss et al., 1976; Pingoud et al., 1975) makes the possibility remote that observations about one monomeric synthetase will have general applicability to the entire class of enzymes.

Nevertheless, the kinetic patterns similar to those observed in this study have been seen for three other aminoacyl-tRNA synthetases: leucyl-, seryl-, and valyl-tRNA synthetases from *E. coli* (Myers et al., 1971). The observed Lineweaver–Burk plots for varied substrates were qualitatively identical with those obtained in this study. These authors interpreted these patterns as indicative of an ordered bi-uni-uni-bi Ping Pong mechanism (eq 7). They attributed the intersecting lines of



(7)

Lineweaver–Burk plots obtained when amino acid and tRNA were the varied substrates to inhibition of the reaction by ATP. The mechanism given by eq 6 did not require postulating inhibition of isoleucyl-tRNA synthetase by ATP nor was any inhibition of isoleucyl-tRNA synthetase by ATP observed during the course of this study. Further, the observed inhibition patterns in the presence of blue dextran are not consistent with an ordered bi-uni-uni-bi Ping Pong mechanism for isoleucyl-tRNA synthetase (Moe & Piszkiewicz, 1979). Thus, our conclusions contrast with the different interpretation of similar data presented by Myers et al. (1971). In spite of differences in interpretation, the similar data obtained for leucyl-, seryl-, valyl-, and isoleucyl-tRNA synthetase from *E. coli* suggest that these enzymes may share a common mechanism.

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Isoleucyl Transfer Ribonucleic Acid Synthetase. Competitive Inhibition with Respect to Transfer Ribonucleic Acid by Blue Dextran†

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ABSTRACT: The inhibitory effects of blue dextran and a small dye molecule derived from it (F3GA-OH) on the steady-state reaction catalyzed by *Escherichia coli* isoleucyl-tRNA synthetase have been studied. Blue dextran gave uncompetitive inhibition with respect to Mg·ATP, mixed inhibition with respect to L-isoleucine, and competitive inhibition with respect to tRNA. The small dye molecule (F3GA-OH) was also competitive with respect to tRNA. These inhibition patterns were not consistent with the bi-uni-uni-bi Ping Pong mechanism generally accepted for aminoacyl-tRNA synthetases. They were consistent with a mechanism in which a second L-isoleucine is bound after isoleucyl-AMP synthesis and before transfer of the isoleucyl moiety to tRNA. Enzyme-bound L-isoleucine lowered the affinity of the enzyme for blue dextran approximately fivefold, a value comparable to the ninefold lowering of the enzyme's affinity for tRNA upon binding L-isoleucine. The affinity of the synthetase for F3GA-OH (K_I = 1.0 × 10^{-7} M) is approximately fivefold higher than its affinity for blue dextran (K_I = 5.3 × 10^{-7} M). These results indicate that blue dextran and its derivatives may be useful for kinetic and physical studies of polynucleotide binding sites on proteins as well as NAD and ATP sites.

The mechanism of action and active-site topography of isoleucyl-tRNA synthetase of *Escherichia coli* (EC 6.1.1.5) have been probed through the use of many inhibitors of its catalytic reaction. These inhibitors have included analogues of L-isoleucine such as its methyl and ethyl esters, 2-methyl-1-butylamine and L-isoleucinol (Holler et al., 1973). Aminoalkyl adenylates, compounds in which the mixed anhydride of the aminoacyl adenylate has been replaced by an ester bond (Cassio et al., 1967), have been extensively studied in their interactions with isoleucyl-tRNA synthetase. Chemically modified tRNAs have been used as competitive inhibitors of isoleucyl-tRNA synthetase with respect to $tRNA^{Ile}$ (Baldwin & Berg, 1966; McNeil & Schimmel, 1972). Also, the trinucleotide U-A-G, a sequence derived from a part of the $tRNA^{Ile}$ structure which has been implicated in its binding to the synthetase (Schoemaker & Schimmel, 1977a), has been shown to be a competitive inhibitor of the synthetase with respect to $tRNA^{Ile}$ (Schoemaker & Schimmel, 1977b).

Previously, we have reported (Moe & Piszkiewicz, 1976) that isoleucyl-tRNA synthetase could be purified by affinity chromatography on blue dextran–Sepharose, presumably due to an attraction of the blue chromophore to a nucleotide binding site of the enzyme (Thompson et al., 1975). In this study we have used blue dextran and the dye molecule derived from it as dead-end inhibitors of the reaction catalyzed by isoleucyl-tRNA synthetase. The results we now report summarize our use of these inhibitors in analyzing the enzyme mechanism, including the finding that the chromophore of blue dextran is competitive for isoleucyl-tRNA synthetase with respect to tRNA.

Materials and Methods

Nomenclature. Cibacron Blue F3GA and Derivatives. Cibacron Blue F3GA is a sulfonated, polyaromatic blue dye derived from 2,4,6-trichloro-*s*-triazine (Thompson et al., 1975). The commercially available dye is the monochlorotriazine derivative (Figure 1) which is used in the manufacture of blue dextran 2000. If this dye is incubated in 0.01 N NaOH at 60 °C, it is hydrolyzed. The expected product of this reaction would be the derivative of Cibacron Blue F3GA in which the chloride is replaced by either a hydroxyl group or its keto tautomer. In this report the Cibacron Blue F3GA and its hydrolysis product will be referred to as F3GA-Cl and F3GA-OH, respectively. The use of F3GA-OH to designate the hydrolysis product is not meant to imply that the molecule

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