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Mechanism of Aminoacylation of tRNA. Proof of the Aminoacyl Adenylate Pathway for the Isoleucyl- and Tyrosyl-tRNA Synthetases from *Escherichia coli* K12†

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ABSTRACT: The following observations show that the formation of isoleucyl-tRNA catalyzed by the isoleucyl-tRNA synthetase from *Escherichia coli* K12 involves the initial rapid formation of an isoleucyl adenylate complex followed by the slow, rate-determining, transfer of the isoleucyl moiety to tRNA. (1) The rate constant for the transfer of [^{14}C]Ile from the E·[^{14}C]Ile~AMP complex to tRNA is the same as the turnover number for the steady-state isoleucylation of tRNA at pH 7.78 (1.5 s^{-1}) and pH 5.87 (0.34 s^{-1}). (2) On mixing a solution of isoleucyl-tRNA synthetase and tRNA with [^{14}C]Ile and ATP the steady-state rate of isoleucylation is attained in the first turnover of the enzyme, with little or no "burst" or charging that would indicate a slow step after the transfer step. (3) The pyrophosphate exchange reaction in the presence of tRNA is 40 times faster than the overall rate of isoleucylation of tRNA. (4) Similarly, rapid quenching experiments indicate that isoleucyl adenylate is formed prior to the transfer step. The possibility that isoleucyl adenylate formation is a parallel reaction caused by a second active site on the enzyme is ruled out both by the stoichiometry in this rapid quenching experiment and also the overall stoichiometry of isoleucyl-tRNA formation. At saturating reagent concentrations the major species in solution is the E·tRNA·Ile~AMP complex. Similar observations are found for the tyrosyl-tRNA synthetase except that at saturating reagent concentrations the rate constants for both tyrosyl adenylate formation and transfer are similar so that both processes contribute to the rate-determining step.

The mechanism of the aminoacylation of tRNA is a subject of some controversy. The classical mechanism, which involves the initial rapid formation of an aminoacyl adenylate complex followed by the transfer of the aminoacyl moiety to the tRNA, has been reviewed by Loftfield (1972) who argues in favor of a "concerted" mechanism in which tRNA, amino acid, and ATP react simultaneously with no intermediate being formed.

One of the arguments against the classical mechanism is that the rate constants for the transfer of the aminoacyl moiety from the E·AA~AMP complexes are too low to account for the observed turnover numbers for the aminoacylation of tRNA. However, these measurements have always been made under conditions that are far from optimal or those found in vivo.

A strategy for determining the mechanistic pathway is to measure: (a) the rate of transfer of the amino acid from the aminoacyl adenylate complex to tRNA to see if it is compatible with the stepwise mechanism; (b) the rate of formation of aminoacyl adenylate in the presence of tRNA, and the rate of aminoacylation of tRNA to see if the aminoacyl adenylate step is faster than the aminoacylation. There are two caveats. First, it is not sufficient in b to measure the steady-state rate of aminoacylation of tRNA since the rate-determining step in this process might be subsequent to the chemical aminoacylation step, for example, the rate-determining diffusion of the charged tRNA from the enzyme as has been found for the isoleucyl-tRNA synthetase from *Escherichia coli B* (Yarus and Berg, 1969; Eldred and Schimmel, 1972). Second, as it has now been demonstrated that even the monomeric aminoacyl-tRNA synthetases have at least two potentially active sites (Fersht, 1975), it must also be shown that the observed rates of aminoacyl adenylate formation and aminoacylation are not occurring separately and concurrently at two different sites.

We have made progress in this direction by introducing new techniques for measuring these rates and applying

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them to the tyrosyl-tRNA synthetase from *E. coli* K12 (Fersht and Jakes, 1975). It was shown that the transfer of the tyrosyl residue from the E·Tyr~AMP complex is faster than the aminoacylation rate constant and that a fraction of the reaction proceeds through the classical pathway. However, a large proportion occurs through a ternary complex, E·tRNA·Tyr·ATP, by an unknown mechanism. In the present study we demonstrate that the aminoacylation of tRNA by the isoleucyl-tRNA synthetase from *E. coli* K12 occurs by the aminoacyl adenylate pathway and this is also the mechanism for the ternary complex route for the tyrosyl-tRNA synthetase.

Experimental Procedure

Materials and Apparatus

Purification of Enzyme. All steps were performed at 4 °C using buffers containing 0.1 mM phenylmethanesulfonyl fluoride and 10 mM mercaptoethanol. The first four steps—namely (1) the rupturing of 50 kg of cells of *E. coli* EM 20031 (a K12 strain) using a Manton Gaulin homogenizer followed by centrifugation; (2) batchwise adsorption of the protein onto DEAE-cellulose (DE 23) at 50 mM potassium phosphate (pH 6.8) and elution with 300 mM phosphate; (3) gradient elution from DE-Sephadex (A-50) with pH 8 phosphate buffer (the enzyme activity elutes at approximately 240 mM phosphate); and (4) gradient elution from hydroxylapatite with pH 6.8 phosphate (enzyme elutes at approximately 250 mM phosphate)—were performed as part of a large scale aminoacyl-tRNA synthetase preparation and will be described in detail elsewhere (Jakes et al., 1976). At this stage the enzyme was approximately 30% of the total protein present. Purification to homogeneity was accomplished by an additional two stages.

Step 5: Gradient Elution on DEAE Sephadex. Protein (800 mg) dissolved in a 100 mM Tris buffer (pH 7.5, 81.25 mM Tris-Cl, 18.75 mM Tris, 50 mM NaCl) was applied to a column (7 × 3.8 cm) of DEAE Sephadex (A-50) equilibrated with the same buffer. After washing with this buffer (250 ml) the column was eluted with a linear gradient of chloride (600 ml, 0.1 M Tris, 100–250 mM NaCl). The enzyme activity was eluted at about 220 mM total chloride concentration. The peak fractions (74–90% pure according to the nitrocellulose filter assay) containing 115 mg of enzyme were concentrated to 15 ml using a 400-ml Amicon with a PM 30 membrane. (The side fractions, containing 100 mg of enzyme, were also collected and subjected to other purification procedures.)

Step 6: Gel Filtration. The concentrated sample was subjected to gel filtration on a column (5 × 88 cm) containing Sephadex G-150 equilibrated in 100 mM Tris-Cl (pH 7.5). The peak fractions containing 70 mg of protein (100% pure) were concentrated as above and dialyzed against 100 mM Tris-Cl (pH 7.5), 50% glycerol, 10 mM mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride. The enzyme was stored at −20 °C.

During preliminary purification experiments the following chromatographic behavior was observed: the enzyme eluted from (a) aminohexyl (AH) Sepharose at 100 mM Tris-Cl (pH 7.3) and about 260 mM NaCl; (b) DEAE Sephadex (A-50) at 200 mM potassium phosphate (pH 6.8).

Unfractionated tRNA was obtained from the Microbiological Research Establishment, Porton, Wilts., England. This had an isoleucine acceptance of 50 pmol/A_{260}. Isoleucine and valine were recrystallized from ethanol–water. All other materials and apparatus have been described previously (Fersht et al., 1975a,b; Fersht, 1975; Fersht and Jakes, 1975).

Methods

All experiments were performed at 25 °C in a standard buffer containing 10 mM $MgCl_2$, 10 mM mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride, and either 144 mM Tris-Cl or 13 mM bis-tris-Cl[1] at pH 5.87.

Active-Site Titrations. (a) ATP Depletion (Fersht et al., 1975a). This was performed either as previously described using approximately 2 μM enzyme and 8 μM ATP or by using the following modification which is somewhat more accurate.

(b) Phosphate Burst. A solution of enzyme (1–2 μM), [γ-^{32}P]ATP (10 μM), isoleucine (500 μM), and inorganic pyrophosphatase was incubated in the standard pH 7.78 buffer. Aliquots (50 μl) were withdrawn at 1-min intervals and quenched into vials containing a solution (200 μl) of 1% charcoal, 0.5 N HCl, and 5 mg of KH_2PO_4. After centrifuging in a Beckman "Microfuge" for 2 min, 50–150 μl samples of the supernatant were assayed for [^{32}P]orthophosphate either by using a water miscible scintillant or directly by Cerenkov radiation. Blanks were performed replacing the enzyme by buffer. End points were measured to calibrate the [^{32}P]phosphate release in relation to the known initial [γ-^{32}P]ATP concentration.

(c) Nitrocellulose Filter Assay (Yarus and Berg, 1970). A solution (60 μl) of the enzyme (0.5–2 μM) containing either of the standard buffers, [^{14}C]Ile (340 Ci/mol, 5–15 μM), ATP (0.5–2 mM), and inorganic pyrophosphatase (0.01–0.1 unit) was incubated at 0 °C for 1 min. Duplicate aliquots (20 μl) were spotted onto nitrocellulose filters (which had been presoaked in bis-tris-Cl at pH 5.87) and then washed with 3.0 ml of cold bis-tris-Cl (pH 5.87). After drying the complex was assayed by scintillation spectrophotometry.

Binding of Isoleucine. The stoichiometry and dissociation constant for the binding of isoleucine were determined by equilibrium dialysis as described (Fersht, 1975) using 8–70 μM enzyme and 2.5 μM to 1.2 mM [^{14}C]Ile.

Steady-State Kinetic Measurements. The initial rates of aminoacylation of tRNA (unfractionated, 8 μM $tRNA^{Ile}$) with [^{14}C]Ile were determined by the usual trichloroacetic acid precipitation procedure. The reaction mixture contained either of the standard buffers, ATP (1.83 mM), inorganic pyrophosphatase (4 units/ml), and the other reagents as indicated. Aliquots were quenched at 30 and 60 s after initiation.

[^{32}P]Pyrophosphate exchange into ATP was measured by incubating a solution of isoleucine (100 μM) with 2 mM [^{32}P]pyrophosphate, 1.83 mM ATP, and the enzyme in the standard pH 7.78 buffer. [β,γ-^{32}P]ATP was monitored after quenching with perchloric acid and adsorption onto charcoal. Alternatively, the pyrophosphate exchange rate was measured using initially unlabeled pyrophosphate and [γ-^{32}P]ATP. The formation of [^{32}P]pyrophosphate was monitored as described for the active-site titration procedure b.

Rapid Quenching Experiments. The pulsed quenched flow apparatus described by Fersht and Jakes (1975) was used.

[1] Abbreviation used is: bis-tris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

(a) Transfer of [^{14}C]Ile from Enzyme·[^{14}C]Ile~AMP to tRNA. One syringe of the apparatus contained enzyme (1.57 μM), ATP (3.6 mM), [^{14}C]Ile (101 μM, 19.1 cpm/pmol), and inorganic pyrophosphatase (0.5 unit/ml) in the standard pH 5.87 buffer (to form the adenylate in situ). The other syringe contained tRNA (15.6 μM tRNA^{Ile}) in the same buffer, and also 1 mM nonradioactive isoleucine (to suppress the steady-state aminoacylation of tRNA with [^{14}C]Ile). The solutions were mixed and automatically quenched with 5% trichloroacetic acid at the desired time intervals. The precipitate was collected on Whatman GF/C filters and dried and the [^{14}C]Ile-tRNA assayed by scintillation counting. The procedure was repeated at pH 7.78.

The experiments were repeated omitting the isoleucine and ATP and using preformed enzyme bound adenylate (1.57 μM) which had been freed from [^{14}C]Ile and ATP by gel filtration.

(b) Preincubation of the Enzyme and tRNA. One syringe of the apparatus contained enzyme (1.57 μM) incubated with tRNA (15.6 μM tRNA^{Ile}) in the pH 7.78 buffer. The other syringe contained [^{14}C]Ile (101 μM, 15.7 cpm/pmol), ATP (1.8 mM), and inorganic pyrophosphatase (0.5 unit/ml) in the same buffer. The solutions were mixed, quenched, and assayed as above. The procedure was repeated at pH 5.87. Experiments were also performed lowering the final concentration of isoleucine to 4 μM and, separately, the ATP to 34 μM. The rate of release of [^{32}P]pyrophosphate from [^{32}P]ATP was determined using final concentrations of 52 μM isoleucine and 32 and 57 μM ATP (31 and 60 cpm/pmol).

Amino Acid Analyses. Protein samples were hydrolyzed in 6 N HCl in vacuo for 22, 40, and 64 h, with and without prior performic acid oxidation (Hirs, 1956). Analyses were carried out on a Durrum amino acid analyzer D-500. The values for Ser and Thr were corrected for destruction and those for Val, Leu, and Ile for incomplete hydrolysis.

Stoichiometry of aminoacylation of tRNA with respect to ATP consumption was determined by measuring the [^{14}C]Ile-tRNA produced on the addition of ATP to a solution of enzyme, tRNA, [^{14}C]Ile, and inorganic pyrophosphatase as described by Fersht and Jakes (1975).

Results

It is known from gel filtration experiments on a partially purified mixture of aminoacyl-tRNA synthetases from *E. coli* K12 that the isoleucyl-tRNA synthetase activity chromatographs with a mol wt of 110 000 (Fayat et al., 1974). Under the denaturing conditions of sodium dodecyl sulfate–polyacrylamide gel electrophoresis the enzyme which had been purified to homogeneity was found to be a single polypeptide chain as has been shown for the corresponding enzymes from *E. coli* B and MRE600 (Arndt and Berg, 1970; Dureković et al., 1973). The amino acid analyses of the three enzymes are very similar (Table I) but it should be noted that Dureković et al. (1973) have shown that the B and MRE600 strains have different N-termini sequences. Assaying the protein concentration by amino acid analysis, the A_{280} was found to be 1.8 cm^{-1} mg^{-1} ml^{-1}, a value similar to that for the MRE 600 enzyme (1.8; Dureković et al., 1973) but higher than that for the *E. coli* B enzyme (1.24; Berthelot and Yaniv, 1970). Protein concentrations were calculated on the basis of A_{280} = 1.8 and a mol wt of 110 000.

Stoichiometry of Isoleucine Binding. In the range 2.5 μM to 1.2 mM isoleucine and pH 7.78 the enzyme binds only 1 mol of isoleucine/mol (0.96 ± 0.02, K_{diss} = 6.7 ± 0.6 μM).

Table I: Amino Acid Composition of Isoleucyl-tRNA Synthetases from *E. coli.*

Amino Acid	Composition		
	K12[a]	B[b]	MRE600[c]
Asp	94	99	88
Thr	49	48	46
Ser	52	46	44
Glu	96	99	91
Pro	44	47	47
Gly	81	82	72
Ala	93	100	93
Val	79	77	59
Met	23	24	23
Ile	47	49	48
Leu	81	80	83
Tyr	39	33	32
Phe	36	32	31
His	31	25	26
Lys	59	65	61
Arg	39	49	45
Cys	14	15	14

[a] This study, based on a mol wt of 110 000. [b] Based on a mol wt of 110 000 (Baldwin and Berg, 1966). [c] Based on a mol wt of 102 000 (Dureković et al., 1973).

Stoichiometry of Aminoacyl Adenylate Formation and Active-Site Titration. The stoichiometry of aminoacyl adenylate formation may be calculated from the burst of [^{32}P]phosphate release on mixing [γ-^{32}P]ATP, isoleucine, and inorganic pyrophosphatase with the enzyme (Fersht et al., 1975a,b). If the rate constant for the formation of the complex under these conditions is much faster than the hydrolysis rate then the burst of phosphate is stoichiometrically equal to the amount of aminoacyl adenylate bound to the enzyme. This is so here. At 10 μM ATP the rate constant for the formation is about 1 s^{-1} and that for the hydrolysis is 1.67 × 10^{-3} s^{-1}. The observed stoichiometry is 1.0 mol/mol of enzyme.

The nitrocellulose filter assay gives a slightly lower figure of 0.94. This difference is consistent with the finding that the retention of the adenylate on the filters is only 93–94% efficient. In the following experiments, the operational normalities of the enzyme solutions were determined by the nitrocellulose filter assay and a factor of 1.06 to normalize to the phosphate burst procedure.

Steady-State Kinetics. The data are summarized in Table II. The important points are: (a) the pyrophosphate exchange reaction is about 60 times faster than aminoacylation; (b) the presence of 11 μM tRNA^{Ile} in the pyrophosphate exchange reaction only slightly decreases the rate and slightly increases the K_M values for ATP and isoleucine; (c) there is a single K_M value only, of 5.69 μM, for isoleucine in the concentration range 15 nM < [Ile] < 50 μM for the aminoacylation of tRNA; and (d) in this reaction the K_M for ATP is only 30 μM.

Rapid Quenching Experiments and Pre-Steady-State Kinetics. (1) Transfer of [^{14}C]Ile from E·[^{14}C]-Ile~AMP. On mixing a solution of isoleucyl-tRNA synthetase [^{14}C]Ile~AMP, which had been freed from [^{14}C]Ile and ATP by gel filtration, with a solution of tRNA (15.6 μM tRNA^{Ile}) at pH 7.78, 65% of the [^{14}C]Ile is transferred to the tRNA with a rate constant of 1.47 s^{-1} (Figure 1). A similar result is obtained when the adenylate complex is

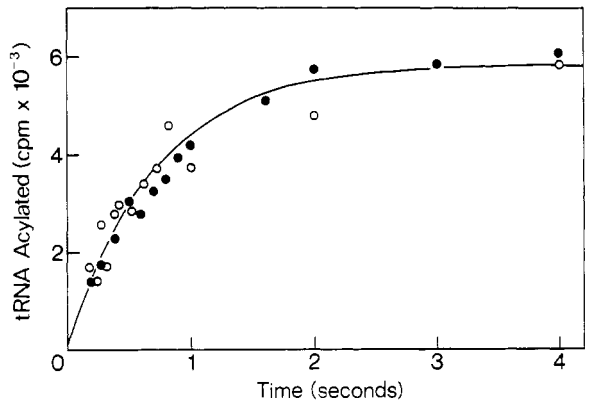


FIGURE 1: Transfer of [^{14}C]Ile from E·[^{14}C]Ile~AMP to tRNA at pH 7.78: (○) one syringe of the pulsed quenched flow apparatus contained the complex (1.57 μM) in a dilute buffer (pH 5.87); the other, tRNA (16 μM) in a concentrated pH 7.78 buffer; (●) one syringe contained 3.6 mM ATP, 101 μM [^{14}C]Ile, inorganic pyrophosphatase, and isoleucyl-tRNA synthetase (1.57 μM) at pH 7.78 to form the complex in situ; the other, tRNA (16 μM) and isoleucine (1 mM) at pH 7.78; as described in the text. The exponential curve is for a rate constant of 1.47 s^{-1}.

Table II: Steady-State Kinetic Data[a] for Isoleucyl-tRNA Synthetase.

Variable Substrate	Constant Substrates and Concns (μM)	K_M (μM)	k_{cat} (s^{-1})
	(a) Aminoacylation of tRNA		
ATP	Ile (54) tRNA (16)	30	1.37
Ile	ATP (1.83 × 10^3) tRNA (16)	5.69	1.46
	(b) Pyrophosphate Exchange (2 mM Pyrophosphate)		
ATP	Ile (50)	730	82
ATP	Ile (50) tRNA (11)	1200	62
Ile	ATP (2 × 10^3)	4	
Ile	ATP (2 × 10^3) tRNA (11)	6	

[a] pH 7.78, 10 mM $MgCl_2$, 25 °C.

formed in situ in the presence of [^{14}C]Ile (104 μM) and ATP (3.6 mM) and inorganic pyrophosphatase, and then mixed with a solution of tRNA that contains 1 mM unlabeled isoleucine to minimize the steady-state charging of tRNA with [^{14}C]Ile. Repetition of these experiments at pH 5.85 shows that 80–88% of the [^{14}C]Ile is transferred with a rate constant of 0.38 s^{-1} for the stripped adenylate and about 0.34 s^{-1} for the adenylate prepared in situ (Figure 2). These first-order rate constants should be compared with the turnover of 1.49 and 0.34 s^{-1} for the steady-state charging of tRNA at pH 7.78 and 5.87.

(2) Preincubation of Enzyme with tRNA. Repetition of the above experiments with the enzyme, preincubated with tRNA, being mixed with ATP and [^{14}C]Ile shows that the steady rate is reached immediately on the first turnover of the enzyme and there is no significant prior "burst" of charging of tRNA (Figure 3). Under the reaction conditions, which are close to saturating reagent concentrations, at pH 7.78 the charging rate is 1.02 ± 0.07 s^{-1} and the "burst" 0.03 ± 0.03 (mol/mol of enzyme), and at pH 5.87 the rate is 0.30 ± 0.01 s^{-1} and the burst 0.006 ± 0.01 (mol/mol of enzyme).

Using [γ-^{32}P]ATP and monitoring the release of pyrophosphate as the tRNA is aminoacylated it is found that at 32 μM [γ-^{32}P]ATP there is a "burst" of 0.23 mol/mol of

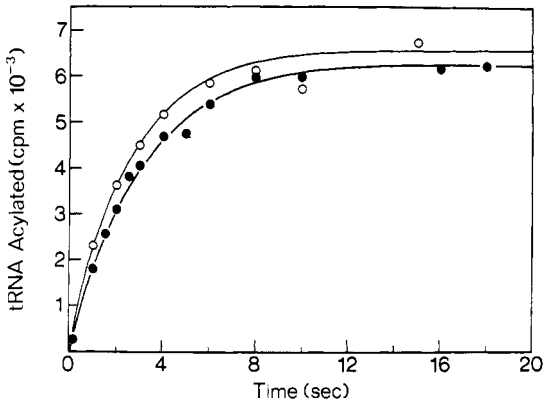


FIGURE 2: Transfer of [^{14}C]Ile from E·[^{14}C]Ile~AMP to tRNA at pH 5.87. As in Figure 1, but pH 5.87 buffer used throughout: (○) preformed "stripped" adenylate complex; (●) complex formed in situ. Exponential curves calculated for rate constants of 0.38 and 0.34 s^{-1}, respectively.

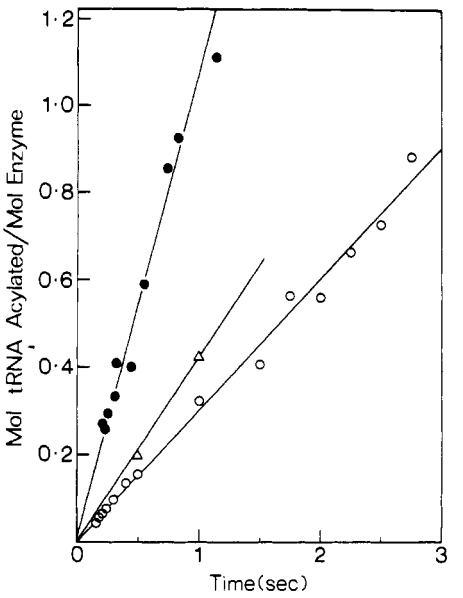


FIGURE 3: Aminoacylation of tRNA during first turnover of enzyme. One syringe of the pulsed quenched flow apparatus contained enzyme (1.57 μM) and tRNA (16 μM) and the other ATP (3.6 mM), inorganic pyrophosphatase, and [^{14}C]Ile (see text): (●) pH 7.78, 101 μM [^{14}C]Ile; (△) pH 7.78, 10 μM [^{14}C]Ile; (○) pH 5.87, 101 μM [^{14}C]Ile. (The rates remain linear during the next few turnovers.)

enzyme with a rate constant of ~2 s^{-1} followed by a linear increase of 0.48 mol/mol of enzyme s^{-1}. At 57 μM [γ-^{32}P]ATP the burst increases to about 0.35 mol/mol, with a subsequent rate of about 0.63 s^{-1} (Figure 4).

It may be shown for the scheme:

$$E\cdot tRNA\cdot AA \overset{ATP}{\rightleftharpoons} E\cdot tRNA\cdot AA\cdot ATP \xrightarrow{fast} E\cdot tRNA\cdot AA\sim AMP + PP_i$$

$$E\cdot tRNA\cdot AA\sim AMP \xrightarrow{slow} E\cdot AA\text{-}tRNA + AMP$$

that the "burst" of pyrophosphate, π, is given by:

$$\pi = [[ATP]/([ATP] + K_M)]^{1/2} \quad (1)$$

where K_M is the observed Michaelis constant for ATP. In this scheme, with a K_M of 30 μM for ATP, "burst" values of 0.27 and 0.41 mol/mol of enzyme are expected for 32 and 57 μM ATP.

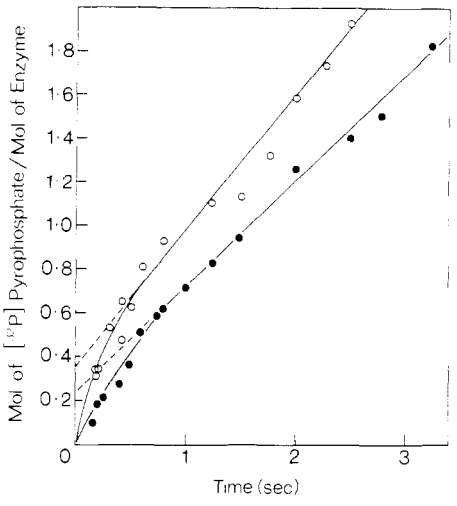


FIGURE 4: Release of [^{32}P]pyrophosphate during aminoacylation. One syringe of the pulsed quenched flow apparatus contained enzyme (1.57 μM), tRNA (16 μM), and inorganic pyrophosphatase (5 units/ml) at pH 7.78; the other isoleucine (101 μM) and either 114 μM (○) or 64 μM (●) [γ-^{32}P]ATP (see text).

The initial rate measurements determined in the pulsed quenched flow apparatus agree within 20 or 30% of those determined by conventional methods using 50–100 times less concentrated enzyme.

Stoichiometry of Isoleucyl-tRNA Formation. Using the method of Fersht and Jakes (1975) it was found that 0.75 mol of [^{14}C]Ile-tRNA is formed per mol of ATP added to a mixture of the enzyme, [^{14}C]Ile, tRNA, and inorganic pyrophosphatase.

Discussion

The following points form the basis for the kinetic arguments. (1) The rate-determining step in the aminoacylation of tRNA is the aminoacylation process itself or a step preceding it. If a subsequent step, such as the diffusion of the charged tRNA from the enzyme, were rate determining, this would lead to the rapid formation of the enzyme bound Ile-tRNA followed by the steady-state turnover rate. This is not observed; the steady-state turnover rate is attained during the first turnover of the enzyme (Figure 3). (2) In the presence of tRNA and [^{32}P]pyrophosphate, exchange of the pyrophosphate into ATP occurs 40 times faster than the rate of aminoacylation of tRNA (k_{cat} = 62 s^{-1} as opposed to 1.5 s^{-1}; Table II). This shows that the enzyme·tRNA complex forms isoleucyl adenylate more rapidly than the rate-determining step in aminoacylation. (3) The rate constant for the transfer of [^{14}C]Ile from the E·[^{14}C]Ile~AMP complex to tRNA is similar to the turnover number for the steady-state aminoacylation of tRNA both at pH 7.78 (1.5 s^{-1}) and pH 5.87 (0.34 s^{-1}) (Figure 4). This is consistent with the rate-determining step in the aminoacylation of the tRNA at saturating concentrations of substrates being the transfer of the amino acid from the aminoacyl adenylate. (4) On mixing enzyme and tRNA with isoleucine and [γ-^{32}P]ATP there is a burst of [γ-^{32}P]pyrophosphate release of the expected magnitude for the reaction conditions and the reaction scheme:

$$\text{E·tRNA} \xrightarrow[\text{ATP, AA}]{\text{fast}} \text{E·tRNA·AA}\sim\text{AMP} \xrightarrow{\text{slow}} \text{AA-tRNA} + \text{E} + \text{AMP} + \text{PP}_i$$

This proves that the major reaction pathway occurs via aminoacyl adenylate formation since the results show that the E·tRNA·AA~AMP complex is relatively rapidly formed and reacts to give AA-tRNA at a rate identical with the turnover number for the overall reaction. At saturating reagent concentrations the E·tRNA·AA~AMP complex is the major solution species.

It could be argued that, perhaps, there are two sets of active sites, and, when tRNA is bound, one active site catalyzes the charging of tRNA through a concerted mechanism while the second rapidly forms the aminoacyl adenylate or catalyzes the pyrophosphate exchange. This argument may be refuted in two ways. First, this would imply in point 4 above that on mixing the enzyme and tRNA with isoleucine and [γ-^{32}P]ATP, one site would release in an exponential manner 1 mol of [^{32}P]pyrophosphate as aminoacyl adenylate is formed and the other would simultaneously further release [^{32}P]pyrophosphate as the tRNA is charged. This is not observed (Figure 4). Second, this would imply that the formation of 1 mol of AA-tRNA would require that at least 2 mol of ATP be hydrolyzed. Again this is not observed. At least 0.75 mol of Ile-tRNA is formed per mol of ATP consumed.

The same arguments may be applied to the aminoacylation of tRNA by the tyrosyl-tRNA synthetase from *E. coli*. We have previously shown that (1) the rate-determining step is not the diffusion of the charged tRNA from the enzyme; (2) the transfer of [^{14}C]Tyr from the E·[^{14}C]-Tyr~AMP complex (28 s^{-1}) is faster than the turnover number for aminoacylation (11 s^{-1}); and (3) 0.97 mol of Tyr-tRNA is formed per mol of ATP consumed (Fersht and Jakes, 1975). We have now shown that under saturating concentrations of reagents, the pyrophosphate exchange reaction in the presence of tRNA (k_{cat} = 23 s^{-1}) is twice as fast as the charging of tRNA in the absence of pyrophosphate (11 s^{-1}). The mechanistic pathway for the aminoacylation of $tRNA^{Tyr}$ also involves an aminoacyl adenylate intermediate.

The isoleucyl-tRNA synthetase from *E. coli* K12 used in this study differs from that from *E. coli* B in that the latter involves the dissociation of the aminoacylated tRNA from the enzyme as the rate-determining step (Yarus and Berg, 1969; Eldred and Schimmel, 1972). It is not inconceivable that the appropriate choice of conditions could lead to this step becoming rate determining also for the K12 enzyme.

Analysis of the K_M Values for ATP and the Amino Acid. In the following scheme:

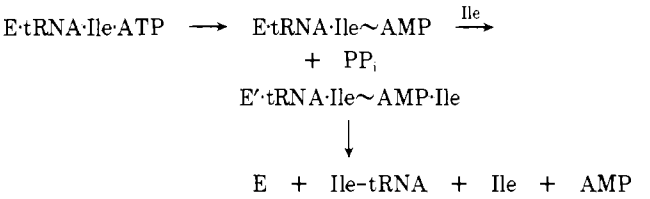
$$\text{E} \underset{\text{S}}{\overset{K_S}{\rightleftharpoons}} \text{ES} \underset{\text{fast}}{\overset{k_2}{\rightarrow}} \text{ES}' \underset{\text{slow}}{\overset{k_3}{\rightarrow}} \text{E} + \text{S}''$$

the K_M for S for the overall production of S″ is much lower than K_S for the first step and is related to this by $K_M \sim K_S k_3/k_2$. (The K_S may be a true dissociation constant or a complex Michaelis constant.) The aminoacylation of tRNA is similar to this scheme where ES′ is the aminoacyl adenylate complex and S″ the charged tRNA. This leads to an apparent anomaly; in general, the k_{cat} for the pyrophosphate exchange reaction ($\equiv k_2$) is much greater than that for the aminoacylation of tRNA but the K_M values for the amino acid and the ATP are often similar for both reactions (Loftfield, 1972). For example, the valyl-tRNA synthetase from *E. coli* B catalyzes the pyrophosphate exchange reaction with a k_{cat} of 41 s^{-1} and K_M values for ATP and valine of 270 and 67 μM, respectively, while the equivalent parameters for aminoacylation are 6 s^{-1}, 1300 μM, and 53 μM

(Yaniv and Gros, 1969). Also, the isoleucyl-tRNA synthetase from *Bacillus stearothermophilus* catalyzes the pyrophosphate exchange reaction in the presence of tRNA 60–100 times faster than aminoacylation but the K_M values for isoleucine are 3.8 and 6.7 μM, respectively (Charlier and Grosjean, 1972). There are three reasons that could account for this in some cases. (a) The pyrophosphate exchange reaction is usually measured in the absence of tRNA. The binding of tRNA to the enzyme may slow down the rate constant for the formation of aminoacyl adenylate so that k_2 is not much greater than k_3. This has been shown to occur for the tyrosyl-tRNA synthetase from *E. coli* (Fersht and Jakes, 1975). (b) Similarly, the binding of tRNA may lower the affinity of the enzyme for the other substrates. (c) The rate-determining step for some of the aminoacylation reactions is the diffusion of the charged tRNA from the enzyme and this step is increased by the binding of a second molecule of amino acid and sometimes ATP, e.g. the isoleucyl- and valyl-tRNA synthetases from *E. coli* B (Yarus and Berg, 1969; Eldred and Schimmel, 1972; Hélène et al., 1971). In this case the observed K_M could be for the binding of the second molecule of amino acid and ATP while another extremely low K_M is undetected. However, none of these arguments apply to the isoleucyl-tRNA synthetase from *E. coli* K12. We have measured both the pyrophosphate exchange rate and the consequent K_M for isoleucine in the presence of tRNA. The exchange rate is 40 times faster than the aminoacylation yet the K_M values for isoleucine are similar for exchange (4 μM) and aminoacylation (5.7 μM) (Table II). This K_M value of 5.7 μM does not represent the stimulation of the dissociation of the Ile-tRNA from the enzyme since this K_M value applies to the first turnover of the enzyme in which dissociation steps would not be manifested (Figure 3). The only way to account for this anomaly is to introduce a new step on the reaction pathway between aminoacyl adenylate formation and aminoacylation which involves the binding of a second molecule of isoleucine. This is not unreasonable. We have shown for the tyrosyl-tRNA synthetase from *B. stearothermophilus* that an E·Tyr~AMP·Tyr·ATP complex is formed and that the Tyr and ATP bind to the enzyme after the production of E·Tyr~AMP. Furthermore, the E·Tyr~AMP complex isolated by gel filtration is not the complex immediately formed on the reaction pathway but is in a different conformational state (Fersht et al., 1975b).

The K_M for ATP in the aminoacylation reaction (30 μM) is considerably less than that for the pyrophosphate exchange reaction in the presence of tRNA (1.2 mM) and is consistent with the scheme involving the rapid accumulation of the adenylate followed by the slow transfer step.

In conclusion, this study shows that (a) an isoleucyl adenylate occurs on the reaction pathway; (b) it is formed more rapidly than the overall rate of aminoacylation of tRNA; (c) the rate-determining step on the reaction pathway is not the dissociation of the charged tRNA from the enzyme but, at saturating reagent concentrations, the transfer of the isoleucyl moiety from the isoleucyl adenylate to the tRNA. Furthermore, it is likely that there is an additional intermediate on the reaction pathway. The following minimal reaction scheme is consistent with the known data for the high concentrations of tRNA, isoleucine, and ATP as found in vivo.



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